<u>DESCRIPTION</u>

MODIFIED STEROID HORMONES FOR GENE THERAPY
AND METHODS FOR THEIR USE

Statement of Rights

The invention described herein was developed in part with funds provided by the National Institutes of Health, Grant Number HD07857. The Government has certain rights.

5   ### Related Applications

This application relates to U.S. patent application Serial No. 60/029,964, filed October 29, 1996, entitled "MODIFIED STEROID HORMONES FOR GENE THERAPY AND METHODS FOR THEIR USE" by O'Malley et al. (Lyon & Lyon Docket No.
10   222/085) which is incorporated herein by reference in its entirety, including any drawings. This application is also related to copending U.S. Application Serial No. 08/479,913, O'Malley et al., filed June 7, 1995, entitled "Modified Steroid Hormones for Gene Therapy and Methods
15   for Their Use," which is a continuation-in-part of co-pending U.S. Application Serial No. 07/939,246, Vegeto, et al., filed September 2, 1992, entitled "Mutated Steroid Hormone Receptors, Methods for Their Use and Molecular Switch for Gene Therapy," the whole of which (including
20   drawings) are all hereby incorporated by reference in their entirety. In addition, this application is related to U.S. Patent No. 5,364,791, Vegeto, et al., issued November 15, 1994, entitled "Progesterone Receptor Having

C-Terminal Hormone Binding Domain Truncations," and PCT/US93/04399 the whole of which (including drawings) are both hereby incorporated by reference in their entirety.

Introduction

5   This invention relates to gene therapy, whereby modified steroid receptors regulate the expression of genes within tissue.   In particular, the modified steroid receptors contain a DNA binding domain, one or more transregulatory domains, and a ligand binding domain and
10   are capable of binding a non-natural ligand.

Background of the Invention

The following description of the background of the invention is provided to aid in the understanding of the invention but is not admitted to describe or constitute
15   prior art to the invention.

Intracellular receptors are a superfamily of related proteins that mediate the nuclear effects of steroid hormones, thyroid hormone and vitamins A and D (Evans, *Science* 240:889-895 (1988)). The cellular presence of a
20   specific intracellular receptor defines that cell as a target for the cognate hormone. The mechanisms of action of the intracellular receptors are related in that they remain latent in the cytoplasm or nuclei of target cells until exposed to a specific ligand (Beato, *Cell* 56:335-344
25   (1989); O'Malley, et al., *Biol. Reprod.* 46:163-167 (1992)). Interaction with hormone then induces a cascade of molecular events that ultimately lead to the specific

association of the activated receptor with other proteins or regulatory elements of target genes.   The resulting positive or negative effects on regulation of gene transcription are determined by the cell-type and promoter-

5 context of the target gene.

In the case of steroid hormones and steroid receptors, such complexes are responsible for the regulation of complex cellular events, including activation or repression of gene transcription.   For example, the ovarian

10 hormones, estrogen and progesterone, are responsible, in part, for the regulation of the complex cellular events associated with differentiation, growth and functioning of female reproductive tissues.   Likewise, testosterone is responsible for the regulation of complex cellular events

15 associated with differentiation growth and function of male reproductive tissues.

In addition, these hormones play important roles in development and progression of malignancies of the reproductive endocrine system.   The reproductive steroids

20 estrogen, testosterone, and progesterone are implicated in a variety of hormone-dependent cancers of the breast (Sunderland, et al., *J. Clin. Oncol.* 9:1283-1297 (1991)), ovary (Rao, et al., *Endocr. Rev.* 12:14-26 (1991)), endometrium (Dreicer, et al., *Cancer Investigation* 10:27-41,

25 (1992)), and possibly prostate (Daneshgari, et al., *Cancer* 71:1089-1097 (1993)).   In addition, the onset of postmenopausal osteoporosis is related to a decrease in production of estrogen (Barzel, *Am. J. Med.* 85:847-850 (1988)).

In addition, corticosteroids are potent and well-documented mediators of inflammation and immunity. They exert profound effects on the production and release of numerous humoral factors and the distribution and prolif-

5   eration of various cellular components associated with the immune and inflammatory responses. For example, steroids are able to inhibit the production and release of cyto-kines (IL-1, IL-2, IL-3, IL-6, IL-8, TNF-α, IFN-γ), chemical mediators (eicosinoids, histamine), and enzymes

10   (MMPs) into tissues, and directly prohibit the activation of macrophages and endothelial cells. Due to the global down-regulation of these physiological events, cortico-steroids have a net effect of suppressing the inflammatory response and have therefore been used extensively to treat

15   a variety of immunological and inflammatory disorders (rheumatoid arthritis, psoriasis, asthma, allergic rhini-tis, etc.).

Besides the therapeutic benefits, however, there are some severe toxic side effects associated with steroid

20   therapy. These include peptic ulcers, muscle atrophy, hypertension, osteoporosis, headaches, etc. Such side effects have hindered the utilization of steroids as therapeutic agents.

In general, the biological activity of steroid hor-

25   mones is mediated directly by a hormone and tissue-specif-ic intracellular receptor. Ligands are distributed through the body by the hemo-lymphatic system. The hormone freely diffuses across all membranes but manifests

its biological activity only in those cells containing the tissue-specific intracellular receptor.

In the absence of ligand, the inactive steroid hormone receptors such as the glucocorticoid ("GR"),
5 mineral corticoid ("MR"), androgen ("AR") progesterone ("PR") and estrogen ("ER") receptors are sequestered in a large complex consisting of the receptor, heat-shock proteins ("hsp") 90, hsp70 and hsp56 and other proteins as well (Smith, et al., *Mol. Endo.* 7:4-11 (1993)). The
10 cellular localization of the physiologically inactive form of the oligomeric complex has been shown to be either cytoplasmic or nuclear (Picard, et al., *Cell Regul.* 1:291-299 (1992); Simmons, et al., *J. Biol. Chem.* 265:20123-20130 (1990)).

15 Upon binding its agonist or antagonist ligand, the receptor changes conformation and dissociates from the inhibitory heteromeric complex (Allan, et al., *J. Biol. Chem.* 267:19513-19520 (1992); Allan, et al., *P.N.A.S.* 89:11750-11754 (1992)). In the case of GR and other
20 related systems such as AR, MR, and PR, hormone binding elicits a dissociation of heat shock and other proteins and the release of a monomeric receptor from the complex (O'Malley, et al., *Biol. Reprod.* 46:163-167 (1992)). Studies from genetic analysis and *in vitro* protease
25 digestion experiments show that conformational changes in receptor structure induced by agonists are similar but distinct from those induced by antagonists (Allan, et al., *J. Biol. Chem.* 267:19513-19520 (1992); Allan, et al., *P.N.A.S.* 89:11750-11754 (1992); Vegeto, et al., *Cell*

69:703-713 (1992)). However, both conformations are incompatible with hsp-binding.

Following the conformation changes in receptor structure, the receptors are capable of interacting with
5   DNA. Studies suggest that the DNA binding form of the receptor is a dimer. In the case of GR homodimers (Tsai, et al., *Cell* 55:361-369 (1988)), this allows the receptor to bind to specific DNA sites in the regulatory region of target gene promoters (Beato, *Cell* 56:335-344 (1989)).
10 These short nucleotide sketches are arranged as palindromic, inverted or repeated repeats (*Id.*). Specificity is determined by the sequence and the spacing of the repeated sequences (Tsai and O'Malley, *Ann. Rev. Biochem.* 63:451-486 (1994)). Following binding of the
15 receptor to DNA, the hormone is responsible for mediating a second function that allows the receptor to interact specifically with the transcription apparatus. Such interaction could either provide positive or negative regulation of gene expression, *i.e.*, steroid receptors are
20 ligand-binding transcription factors, capable of not only activating but also repressing the expression of specific genes. Studies have shown, however, that repression does not require DNA binding.

For instance, when bound to their intracellular
25 receptors, corticosteroids can affect the transcription of a variety of genes whose products play key roles in the establishment and progression of an inflamed situation. Such genes include those encoding for cytokines, chemical mediators and enzymes. Transcription of these genes can

be repressed or activated depending on the transcription factors and/or regulatory sequences controlling the expression of the gene. Presently there are numerous reports documenting the effect of glucocorticoid on the
5   expression of various genes at the transcriptional level.

In particular, the glucocorticoid receptor is a member of a family of ligand-dependent transcription factors capable of both positive and negative regulation of gene expression (Beato, *FASEB J.* 5:2044-2051 (1991);
10   Pfahl, *Endocr. Rev.* 14:651-658, (1993); Schule, et al., *Trends Genet.* 7:377-381 (1991)). In its inactivated form, the GR is part of a large heteromeric complex which includes hsp90 as well as other proteins (Denis, et al., *J. Biol. Chem.* 262:11803-11806 (1987); Howard, et al., *J.*
15   *Biol. Chem.* 263:3474-3481 (1988); Mendel, et al., *J. Biol. Chem.* 261:3758-3763 (1986); Rexin, et al., *J. Biol. Chem.* 267:9619-9621 (1992); Sanchez, et al., *J. Biol. Chem.* 260:12398-12401 (1985)), and hsp56 (Lebea, et al., *J. Biol. Chem.* 267:4281-4284 (1992); Pratt, *J. Steroid*
20   *Biochem. Mol. Biol.* 46:269-279 (1993); Sanchez, *J. Biol. Chem.* 265:22067-22070 (1990); Yem, *J. Biol. Chem.* 267:2868-2871, (1992)). Binding of agonist stimulates receptor activation, dissociation from hsp90 and the other proteins (Denis, et al., *Nature* 333:686-688 (1988);
25   Sanchez, et al., *J. Biol. Chem.* 262:6986-6991 (1987)), and nuclear translocation, prerequisites for both transactivation and transrepression.

Cloning of several members of the steroid receptor superfamily has facilitated the reconstitution of hormone-

dependent transcription in heterologous cell systems and facilitated delineation of the GR activation and repression mechanisms. Subsequently, *in vivo* and *in vitro* studies with mutant and chimeric receptors have
5   demonstrated that steroid hormone receptors are modular proteins organized into structurally and functionally defined domains. Deletion mutants of the GR have determined that the transactivation domain is located at the N-terminal amino acid sequence positioned between
10 amino acids 272 and 400 (Jonat, et al., *Cell* 62:1189-1204 (1990)). A well defined 66 amino acid DNA binding domain ("DBD") has been identified and studied in detail, using both genetic and biochemical approaches (Lucibello, et al., *EMBO J.* 9:2827-2834 (1990)). The ligand or hormone
15 binding domain ("LBD"), located in the carboxyl-terminal portion of the receptor, consists of about 300 amino acids (Kerppola, et al., *Mol. Cell. Biol.* 13:3782-3791 (1993)). The LBD has not been amenable to detailed site-directed mutagenesis, since this domain appears to fold into a
20 complex tertiary structure, creating a specific hydrophobic pocket which surrounds the effector ligand when bound. This feature creates difficulty in distinguishing among amino acid residues that affect the overall structure of the LBD domain from those involved in
25 a direct contact with the ligand. The LBD also contains sequences responsible for receptor dimerization, nuclear localization, hsp interactions and transactivation sequences of the receptor (Fuller, et al, *FASEB J.* 5:3092-3099 (1991)).

The mechanism of gene activation is generally better understood than that of repression. For transactivation, a ligand-induced conformational change, comparable to that inferred to be necessary for activation of the

5   progesterone (Allan, et al., *Proc. Natl. Acad. Sci. USA* 89:11750-11754 (1992)) and estrogen (Beekman, et al., *Mol. Endocrinol.* 7:1266-1274 (1993)) receptors, is required for efficient activation of the transcription activating function of the receptor (Hollenberg and Evans, *Cell*

10 55:899-906 (1988); Webster, et al., *Cell* 54:199-207, (1988)).   Furthermore, the conformational change is required for interaction of the receptor with other components of the transcription apparatus.   Transactivation is mediated by a receptor dimer bound to a

15 glucocorticoid response element ("GRE").   Such transactivation occurs exclusively by homodimerization. This is mainly achieved by a region in the second zinc finger of the receptor known as the D-loop (Umesono, et al., *Cell* 57:1139-1146 (1989); Dahlman-Wright, et al., *J.*

20 *Biol. Chem.* 266:3107-3112 (1991)).   The resulting homodimers then bind to the palindromic GRE to initiate the transcriptional activation process (Evans, *Science* 240:889-895 (1988); Cato, et al., *J. Steroid Biochem. Mol. Biol.* 43:63-68 (1992)).

25   Transrepression, on the other hand, appears to be mediated by the monomeric form of the receptor through interactions with other transcriptional factors, including AP-1 and NF_K-B, preventing them from carrying out their function as transcriptional activators (Hoeck, et al.,

EMBO   J.   13:4087-4095   (1994)).   Studies also show transrepression by the dimeric form of the receptor.   In the case of the monomeric pathway, studies suggest that AP-1 prevents hormone-dependent activation of GR-regulated

5 promoters through a mutually inactive complex formed either by a direct protein-protein interaction of the receptor and AP-1 or through a third partner (Miner, et al., *Cell Growth Differ.* 2:525-530   (1991);   Pfahl, *Endocrine Rev.* 14:651-658 (1993)). Such transrepression

10 of AP-1 and NF_K-B mediated by the monomeric form of the receptor depends on the presence of the DNA binding domain. It does not depend on the ability of the receptor to bind DNA.   In the case of the dimeric form of the receptor, several studies suggest mechanisms for such GR-

15 mediated transrepression include GR binding to a sequence overlapping a *cis*-acting element for another *trans*-acting factor, thereby displacing it from, or preventing its binding to, its cognate element (Akerblom, et al., *Science* 241:350-353 (1988); Drouin, et al., *Mol. Cell. Biol.*

20 9:5305-5314   (1989);   Oro,   et   al.,   *Cell*   55:1109-1114, (1988); Stromstedt, et al., *Mol. Cell. Biol.* 11:3379-3383, (1991)).

As   noted   above,   GR-mediated   transrepression attributed to direct or indirect interaction of the GR

25 with other *trans*-acting factors, results in inhibition of their activity and/or ability to bind to DNA (Celada, et al., *J. Exp. Med.* 177:691-698 (1993); Diamond, et al., *Science* 249:1266-1272 (1990); Gauthier, et al., *Embo J.* 12:5089-5096 (1993); Jonat, et al., *Cell* 62:1189-1204

(1990); Kutoh, et al., *Mol. Cell Biol.* 12:4955-4969
(1992); Lucibello, et al., *Embo J.* 9:2827-2834 (1990);
Ray, et al., *Proc. Natl. Acad. Sci. USA* 91:752-756 (1994);
Schule, et al., *Cell.* 62:1217-1226 (1990); Tverberg, et
5 al., *J. Biol. Chem.* 267:17567-17573 (1992); Yang-Yen, et
al., *Cell* 62:1205-1215 (1990); Lucibello, et al., *EMBO J.*
9:2827-2834 (1990)). These models require ligand binding
to stimulate receptor activation, dissociation from hsp90,
and nuclear translocation. It is not clear whether these
10 mechanisms are dependent on the same ligand-induced
conformational change needed for transactivation.
However, a transactivation-defective mutant represses the
AP-1 dependent promoter suggesting that the
transactivation function of the receptor is not required
15 for the repression of AP-1 activity (Yang-Yen, et al.,
Cell 62:1205-1215 (1990)). Furthermore, similar studies
also suggest that the transactivation function of the
receptor is not required for the repression of NF_K-B
activity.
20   In attempts to decipher the transrepression
mechanism, studies have reviewed the role of the bound
ligand in GR-mediated repression of AP-1-responsive genes
containing a tetradecanoyl phorbol acetate ("TPA")
response element. Repression of these genes has been
25 proposed to be the result of the direct interaction of the
GR with c-Jun (Diamond, et al., *Science* 249:1266-1272
(1990); Lucibello, et al., *EMBO J.* 9:2827-2834 (1990);
Schule, et al., *Cell* 62:1217-1226 (1990); Touray, et al.,
Oncogene 6:1227-1234 (1991); Yang-Yen, et al., *Cell*

62:1205-1215 (1990)) or c-Fos (Kerppola, et al., *Mol. Cell. Biol.* 13:3782-3791 (1992)) which are components of the AP-1 transcription complex. The GR DNA-binding domain is necessary for this interaction, since most mutations in

5   this domain result in the loss of repressor activity *in vivo* (Diamond et al., *Science* 249:1266-1272 (1990); Jonat et al., *Cell* 62:1189-1204 (1990); Lucibello et al., *EMBO J.* 9:2827-2834 (1990); Schule et al., *Cell* 62:1217-1226 (1990); Yang-Yen et al., *Cell* 62:1205-1215 (1990)).

10   The DNA-binding domain is also necessary for inhibition of *in vitro* transcription from the collagenase promoter and inhibition of Jun-Fos heterodimer binding to the collagenase TPA response element (Mordacq et al., *Genes Dev.* 3:760-769 (1989)). However, deletion or

15   truncation of the ligand-binding domain also results in a significant loss of repressor activity (Jonat et al., *Cell* 62:1189-1204 (1990); Schule et al., *Cell* 62:1217-1226 (1990); Yang-Yen et al., *Cell* 62:1205-1215 (1990)), suggesting that the ligand-binding domain may contribute

20   to, or modulate, the inhibition of AP-1 activity.

Further studies examining the role of the ligand in GR-mediated transrepression of the collagenase promoter found efficient receptor-mediated transrepression with ligand-free mutant GR in which the first cysteine residue

25   of the proximal zinc finger was replaced with tyrosine (Liu et al., *Mol. Cell. Bio.* 15:1005-1013 (1995)). Such studies suggest that neither retention of the ligand nor direct binding of the receptor to DNA is required, *i.e.*,

that transrepression of AP-1 activity by GR is ligand independent.

The expression of most mammalian genes is intricately regulated *in vivo* in response to a wide range of stimuli, including physical (pressure, temperature, light), electrical (e.g. motor and sensory neuron signal transmission) as well as biochemical (ions, nucleotides, neurotransmitters, steroids and peptides) in nature. While the mechanism of transcriptional regulation of gene expression has been extensively studied (McKnight, *Genes Dev.* 10:367-381 (1996)), progress on achieving target gene regulation in mammalian cells, without interfering with endogenous gene expression, has been limited. Currently, most strategies for target gene activation or repression are performed in a constitutive manner. Such uncontrolled regulation of gene expression is not ideal physiologically, and can even be deleterious to cell growth and differentiation. In contrast, use of the yeast GAL4 DNA binding domain in this invention does not interfere with endogenous genes since that chimeric regulator will only recognize target gene constructs containing the GAL4 binding sequence.

Several inducible systems have been employed for controlling target gene expression. These inducible agents include heavy metal ions (Mayo et al., *Cell* 29:99-108 (1982)), heat shock (Nover et al. *CRC Press* 167-220 (1991)), isopropyl β-D-thiogalactoside (β-gal) (Baim et al. *Proc. Natl. Acad. Sci.* 88:5072-5076 (1981)), and steroid hormones such as estrogen (Braselmann et al. *Proc.*

Natl. Acad. Sci. 90:1657-1661 (1993)) and glucocorticoids (Lee et al. *Nature* 294:228-232 (1981)). However, many of these inducers are either toxic to mammalian cells or interfere with endogenous gene expression (Figge et al.

5   *Cell* 52:713-722 (1988)).

Utilizing a bacterial tetracycline-responsive operon element, Gossen *et al.* developed a model for controlling gene expression with a tetracycline-controlled transactivator (tTA and rtTA) (Gossen et al. *Proc. Natl.*

10 *Acad. Sci.* 89:5547-5551 (1992); Gossen et al. *Science* 268:1766-1769 (1995)). No *et al.* recently reported a three-component system consisting of a chimeric GAL4-VP16-ecdysone receptor, its partner retinoid X receptor (RXR), and a target gene. They demonstrated its application in

15 activating reporter gene expression in an ecdysone-dependent manner (No et al. *Proc. Natl. Acad. Sci.* 93:3346-3351 (1996). The invention described herein has advantages over the No and Gossen models, as the chimeric regulator recognizes only the target gene constructs and

20 not endogenous genes, and the system is only activated in the presence of an exogenous compound, but not in the presence of any endogenous molecules.

Summary of the Invention

Construction of novel modified steroid hormone

25 receptors which regulate the expression of nucleic acid sequences is described herein, and surprisingly these modifications allow control of the transactivation and transrepressing functions of the modified steroid hormone

receptor.   Such modifications unexpectedly allow the
receptors to bind various ligands whose structures differ
dramatically from the naturally-occurring ligands (for
example, non-natural ligands, anti-hormones and non-native
5   ligands) and thereby provide a substantial improvement
over prior attempts to control or regulate target gene
expression.

These modifications are generated in the ligand
binding domain of the GR and eliminate the ability of the
10   GR to bind its natural ligand.   These modified steroid
receptors exhibit normal transactivation and transrepres-
sion activity; however, stimulation of such activity
occurs via activation by a non-natural and exogenously or
endogenously applied ligand.   Modifications are also
15   generated in the ligand binding domain of the PR and
eliminate the ability of PR to bind its natural ligand.
Replacement of the GR binding domain with the modified PR
binding domain allows the stimulation of GR responsive
gene expression via non-natural ligands.

20   Other modifications to the GR ligand binding domain
in conjunction with modifications to the DNA binding
domain of GR eliminate the ability of steroid hormones to
initiate transactivation by its natural ligand.   Instead,
such modifications allow the modified receptor to bind
25   non-natural ligands and stimulate the transrepression of
gene expression but not transactivation.   Likewise, using
the same ligand binding domain modification in conjunction
with modifications to the transregulatory domain allows
the modified receptor to bind non-natural ligands and

stimulate transactivation but not transrepression of gene expression.

Other modifications remove the ligand binding domain completely to create a constitutively active steroid

5 receptor. Such modifications cause continual transactivation and transrepression effects on the regulation of gene transcription. In addition, modifications that selectively eliminate either transactivation or transrepression functions are incorporated into the constitutively active

10 steroid receptor thereby constitutively transrepressing or transactivating gene expression. Furthermore, other modifications use a ligand binding domain which recognizes its natural ligand or if modified recognizes a non-natural ligand, but is fused with a DNA binding domain and trans-

15 regulatory domains not associated normally with the ligand binding domain. Such a construct is capable of regulating the expression of a gene not normally associated with the ligand binding domain in a wild type receptor protein.

These modified receptors can be expressed by special-

20 ly designing DNA expression vectors to control the level of expression of recombinant gene products. The steroid receptor family of gene regulatory proteins is an ideal set of such molecules. These proteins are ligand activated transcription factors whose ligands can range

25 from steroids to retinoids, fatty acids, vitamins, thyroid hormones and other presently unidentified small molecules. These compounds bind to receptors and either activate or repress transcription.

These receptors are modified to allow them to bind various ligands whose structure is either naturally occurring or differs from naturally occurring ligands. By screening receptor mutants, receptors can be selected that
5   respond to ligands which do not activate the host cell endogenous receptor. Thus, regulation of a desired transgene can be achieved using a ligand which binds to and regulates a customized receptor. This occurs only with cells that have incorporated and express the modified
10   receptor.

Taking advantage of the abilities of the modified steroid hormone receptor to effect regulation of gene expression, these gene constructs can be used as thera-peutic gene medicines, for gene replacement, and in gene
15   therapy. These modified receptors are useful in gene therapy where the level of expression of a gene, whether transactivation or repression, is required to be controlled. The number of diseases associated with inappropriate production or responses to hormonal stimuli
20   highlights the medical and biological importance of these constructs.

The properties of the modified steroid hormone receptors allow most or all of the deleterious effects of steroids to be avoided while generally maintaining their
25   therapeutic benefits. In particular, administration of steroids typically causes toxicity problems. The delete-rious effects of steroids can be attributed to the *in vivo* transactivation or transrepression of certain genes. These toxic effects may well be the result of both

transactivation and transrepression, or be primarily attributable to one of them. The present invention features the use of modified GR molecules as gene medicines for the replacement of steroid therapy. These

5   synthetic receptors retain functions similar to those of the endogenous receptors, but by responding to alternative ligands, eliminate some of the toxic side effects attributable to currently used steroid therapy.

This ability of the GR constructs to avoid steroid

10 toxicity but still exhibit therapeutic effects allows the constructs to be used for treating numerous diseases, including arthritis, asthma, senile dementia or Parkinson's disease. Furthermore, the constructs can be used for preventing or treating diseases in which inap-

15 propriate production or responses to hormonal stimuli exists, *e.g.*, hormone-dependent cancers of the breast, ovary, endometrium, prostate, and post-menopausal osteo- porosis. The constructs also can be used in conjunction with co-transfected expression vectors so as to operate as

20 a gene switch. For detailed description of gene switch, see, U.S. Application Serial No. 07/939,246, Vegeto et al., and U.S. Patent No. 5,364,791, Vegeto et al., the whole of which (including drawings) are both hereby incorporated by reference.

25   In addition, the constructs above can be used for gene replacement therapy in humans and for creating transgenic animal models used for studying human diseases. The transgenic models can be used as well for assessing and exploring novel therapeutic avenues to treat effects

of chemical and physical carcinogens and tumor promoters. The above constructs can also be used for distinguishing steroid hormone receptor antagonists and steroid hormone receptor agonists. Such recognition of antagonist or

5   agonist activity can be performed using cells transformed with the above constructs. Thus, in view of the above, various aspects of the invention will now be described.

In a first aspect, the present invention features a modified glucocorticoid receptor fusion protein. The

10 fusion protein receptor contains a GR with its ligand-binding domain replaced with a mutated PR ligand-binding domain. This fusion protein is capable of being activated by the binding of a non-natural ligand, but not by natural or synthetic glucocorticoid or other natural or synthetic

15 steroids. The fusion protein includes a glucocorticoid receptor region which comprises a DNA binding domain and one or more transregulatory domains. The transregulatory domains are capable of transactivating or transrepressing glucocorticoid responsive gene expression.

20   In addition to the glucocorticoid receptor region, the fusion protein also includes a mutated progesterone ligand binding region which is capable of binding a non-natural ligand. The mutated ligand binding region is preferably mutated by deletion of about 16 to 42 carboxyl

25 terminal amino acids of a progesterone receptor ligand binding domain. The mutated progesterone receptor ligand binding region preferably comprises, consists essentially of, or consists of about amino acids 640 through 891 of a progesterone receptor. Other preferred embodiments

comprise, consist essentially of, or consist of amino acids 640-917, amino acids 640-920 or amino acids 640-914. One skilled in the art will recognize that various mutations can be created to achieve the desired function.

5   The term "fusion protein" as used herein refers to a protein which is composed of two or more proteins (or fragments thereof) where each protein occurs separately in nature.   The combination can be between complete amino acid sequences of the protein as found in nature, or

10   fragments thereof.   In the case of the glucocorticoid-progesterone fusion protein receptor, the fusion protein is preferably composed of portions of the glucocorticoid receptor and the progesterone receptor. This combination can include the complete amino acid sequence of each

15   protein or fragments thereof.   For example, the glucocorticoid-progesterone fusion protein may include the ligand binding domain of progesterone and the DNA binding domain and transregulatory domains of the glucocorticoid receptor.   This is only an example and not meant to be

20   limiting.

The term "non-natural ligand" as used herein refers to compounds which can normally bind to the ligand binding domain of a receptor, but are not the endogenous ligand. "Endogenous" as used herein refers to a compound

25   originating internally within mammalian cells.   The receptor is not exposed to the ligand unless it is exogenously supplied. "Exogenous" as used herein refers to a compound originating from external sources and not normally present within mammalian cells.   This also

includes ligands or compounds which are not normally found in animals or humans. Non-natural also includes ligands which are not naturally found in the specific organism (man or animal) in which gene therapy is contemplated.

5 These ligands activate receptors by binding to the modified ligand binding domain. Activation can occur through a specific ligand-receptor interaction whether it is through direct binding or through association in some form with the receptor.

10   "Natural ligand" as used here refers to compounds which normally bind to the ligand binding domain of a receptor and are endogenous. The receptor in this case is exposed to the ligand endogenously. Natural ligands include steroids, retinoids, fatty acids, vitamins,

15 thyroid hormones, as well as synthetic variations of the above. This is meant to be only an example and non-limiting.

The term "ligand" as referred to herein means any compound which activates the receptor, usually by

20 interaction with the ligand binding domain of the receptor. Ligand includes a molecule or an assemblage of molecules capable of specifically binding to a modified receptor. The term "specifically binding" means that a labeled ligand bound to the receptor can be completely

25 displaced from the receptor by the addition of unlabeled ligand, as is known in the art.

Examples of non-natural ligands and non-native ligands may be found in PCT Publication PCT/US96/04324,

the whole of which (including drawings) is hereby incorporated by reference.

The term "binding" or "bound" as used herein refers to the association, attaching, connecting, or linking
5   through covalent or non-covalent means, of a ligand, whether non-natural or natural, with a corresponding receptor. The ligand and receptor interact at complementary and specific within sites on a given structure. Binding includes, but is not limited to, components which
10 associate by electrostatic binding, hydrophobic binding, hydrogen binding, intercalation or forming helical structures with specific sites on nucleic acid molecules.

The term "glucocorticoid receptor" refers to a steroid hormone receptor which responds to a glucocorti-
15 coid ligand. The glucocorticoid receptor is part of the steroid hormone receptor superfamily which are known steroid receptors whose primary sequence suggests that they are related to each other. Representative examples of such receptors include the estrogen, progesterone,
20 Vitamin D, chicken ovalbumin upstream promoter trans-factor, ecdysone, Nurr-1 and orphan receptors, glucocorti-coid-α, glucocorticoid-β, mineralocorticoid, androgen, thyroid hormone, retinoic acid, and retinoid X. These receptors are composed of DNA binding domains, ligand
25 binding domains, as well as transregulatory domains.

The glucocorticoid receptor is a ligand-dependent transcription factor capable of both positive and negative regulation of gene expression. Interaction of the receptor with a ligand induces a cascade of molecular

events that ultimately lead to the specific association of the activated receptor with regulatory elements of target genes. In an inactive form such receptors form a large complex comprising the receptor, heat shock proteins and

5   other proteins.

The term "glucocorticoid receptor region" refers to a fragment or part of the complete glucocorticoid receptor as defined above. A glucocorticoid receptor region may retain complete or partial activity of the natural

10 receptor protein. For example, a glucocorticoid receptor region might contain only the DNA binding domain and the transregulatory domains and not the ligand binding domain, or vice versa. This is only an example and not meant to be limiting.

15 The term "ligand binding domain" or "ligand binding region" as used herein refers to that portion of a steroid hormone receptor protein which binds the appropriate hormone or ligand and induces a cascade of molecular events that ultimately leads to the specific association

20 of the activated receptor with regulatory elements of target genes. This includes, but is not limited to, the positive or negative effects on regulation of gene trans-cription. Binding of ligand to the ligand binding domain induces a conformation change in the receptor structure.

25 The conformational change includes the dissociation of heat shock proteins and the release of a monomeric receptor from the receptor complex, as well as a different tertiary or 3-dimensional structure. The conformational

change that occurs is specific for the steroid receptor and ligand that binds to the ligand binding domain.

For example, for glucocorticoid receptors, the conformation change that occurs when glucocorticoid
5 hormone binds allows homodimerization, *i.e.*, dimerization between two identical GR molecules. However, heterodimerization can occur with other steroid receptors, *i.e.*, dimerization with two molecules such as GR and ER. Such dimerization allows the receptor to bind with DNA or
10 induce the regulatory effect by binding other transcription factors.

The term "DNA binding domain" as used herein refers to that part of the steroid hormone receptor protein which binds specific DNA sequence in the regulatory regions of
15 target genes. This domain is capable of binding short nucleotide stretches arranged as palindromic, inverted or repeated repeats. Such binding, will activate gene expression depending on the specific ligand and the conformational changes due to such ligand binding. For
20 repression, DNA binding is not needed.

The term "transregulatory domain" as used herein refers to those portions of the steroid hormone receptor protein which are capable of transactivating or transrepressing gene expression. This would include
25 different regions of the receptor responsible for either repression or activation, or the regions of the receptor responsible for both repression and activation. Such regions are spatially distinct. The above is only an example and meant to be non-limiting. For transrepres-

sion, this domain under one mechanism is involved with dimerization which in turn causes a protein/protein interaction to prevent or repress gene expression. Such regulation occurs when the receptor is activated by the
5   ligand binding to the ligand binding domain. The conformational change of the receptor is capable of forming a dimer with a discrete portion of the transregulatory domain to repress gene expression. In addition, repression can occur through a monomeric form of
10 the receptor, however, DNA binding is not necessary (see below).

The terms "transactivation," "transactivate," or "transactivating" refer to a positive effect on the regulation of gene transcription due to the interaction of
15 a hormone or ligand with a receptor causing the cascade of molecular events that ultimately lead to the specific association of the activated receptor with the regulatory elements of the target genes. Transactivation can occur from the interaction of non-natural as well as natural
20 ligands. Agonist compounds which interact with steroid hormone receptors to promote transcriptional response can cause transactivation. Such positive effects on transcription include the binding of an activated receptor to specific recognition sequences in the promoter of target
25 genes to activate transcription. The activated receptors are capable of interacting specifically with DNA. The hormone- or ligand-activated receptors associate with specific DNA sequences, or hormone response elements, in the regulatory regions of target genes. Transactivation

alters the rate of transcription or induces the transcrip-
tion of a particular gene(s). It refers to an increase in
the rate and/or amount of transcription taking place.

       The terms "transrepress," "transrepression" or
5 "transrepressing" as used herein refer to the negative
effects on regulation of gene transcription due to the
interaction of a hormone or ligand with a receptor
inducing a cascade of molecular events that ultimately
lead to the specific association of the activated receptor
10 with other transcription factors such as NF_K-B or AP-1.
Transrepression can occur from the interaction of non-
natural as well as natural ligands. Antagonist and
agonist compounds which interact with steroid hormone
receptor can cause transrepression. Once the ligand binds
15 to the receptor, a conformational change occurs. Trans-
repression can occur via two different mechanisms, i.e.,
through the dimeric and monomeric form of the receptor.
Use of the monomeric form of the receptor for transrepres-
sion depends on the presence of the DNA binding domain but
20 not on the ability of the receptor to bind DNA. Use of
the dimeric form of the receptor for transrepression
depends on the receptor binding response elements
overlapping cis-element(s). Transrepression alters the
rate of transcription or inhibits the transcription of a
25 particular gene.   Transrepression decreases the rate
and/or the amount of transcription taking place.

       The term "progesterone receptor" as used herein also
refers to a steroid hormone receptor which responds to or
is activated by the hormone progesterone. Progesterone is

part of the steroid hormone receptor superfamily as described above. The progesterone receptor can exist as two distinct but related forms that are derived from the same gene. The process for generation of the products may

5   be alternate initiation of transcription, splicing differences, or transcription termination. These receptors are composed of DNA binding, ligand binding, as well as transregulatory domains. The progesterone receptor is also a ligand-dependent transcription factor capable of

10 regulating gene expression. Interaction of the progesterone receptor with a ligand induces a cascade of molecular events that ultimately lead to the specific association of the activated receptor with regulatory elements of target genes.

15   The term "modified," "modification," "mutant" or "mutated" refers to an alteration of the receptor from its naturally occurring wild-type form. This includes alteration of the primary sequence of a receptor such that it differs from the wild-type or naturally-occurring

20 sequence. The mutant steroid hormone receptor protein as used in the present invention can be a mutant of any member of the steroid hormone receptor superfamily. For example, a steroid receptor can be mutated by deletion of amino acids on the carboxyl terminal end of the protein.

25 Generally, a deletion of from about 1 to about 120 amino acids from the carboxyl terminal end of the protein provides a mutant steroid hormone receptor useful in the present invention. A person having ordinary skill in this art will recognize, however, that a shorter deletion of

carboxyl terminal amino acids will be necessary to create useful mutants of certain steroid hormone receptor proteins. Other mutations or deletions can be made in other domains of the steroid receptor of interest, such as

5   the DNA binding domain or the transregulatory domain.

For example, a mutant of the progesterone receptor protein will contain a carboxyl terminal amino acid deletion of approximately 1 to 60 amino acids. In a preferred embodiment of the present invention, 42 carboxyl

10   terminal amino acids are deleted from the progesterone receptor protein. Likewise, a mutation of one or more amino acids in the DNA binding domain or the trans-regulatory domains can change the regulation of gene expression.

15   One skilled in the art will recognize that a combination of mutations and/or deletions are possible to gain the desired response. This would include double point mutations to the same or different domains. In addition, mutation also includes "null mutations" which

20   are genetic lesions to a gene locus that totally inactivate the gene product.

Examples of mutations are described in PCT Publication PCT/US96/04324, the whole of which (including drawings) is hereby incorporated by reference.

25   The term mutation also includes any other derivatives. The term "derivative" as used herein refers to a peptide or compound produced or modified from another peptide or compound of a similar structure. Such a derivative may be a "chemical derivative," "fragment,"

"variant," "chimera," or "hybrid" of the complex. A derivative retains at least a portion of the function of the protein (for example reactivity with an antibody specific for the complex, enzymatic activity or binding

5 activity mediated through noncatalytic domains) which permits its utility in accordance with the present invention.

A derivative may be a complex comprising at least one "variant" polypeptide which either lacks one or more amino

10 acids or contain additional or substituted amino acids relative to the native polypeptide. The variant may be derived from a naturally occurring complex component by appropriately modifying the protein DNA coding sequence to add, remove, and/or to modify codons for one or more amino

15 acids at one or more sites of the C-terminus, N-terminus, and/or within the native sequence. It is understood that such variants having added, substituted and/or additional amino acids retain one or more characterizing portions of the native complex. A functional derivative of complexes

20 comprising proteins with deleted, inserted and/or substituted amino acid residues may be prepared using standard techniques well-known to those of ordinary skill in the art.

A "chemical derivative" of the complex contains

25 additional chemical moieties not normally a part of the protein. Such moieties may improve the molecule's solubility, absorption, biological half life, and the like.

The term "modified" or "modification" as used herein refers to a change in the composition or structure of the compound or molecule. However, the activity of the derivative, modified compound, or molecule is retained,

5 enhanced, or increased relative to the activity of the parent compound or molecule. This would include the change of one amino acid in the sequence of the peptide or the introduction of one or more non-naturally occurring amino acids or other compounds. This includes a change in

10 a chemical body, a change in a hydrogen placement, or any type of chemical variation. In addition, "analog" as used herein refers to a compound that resembles another structure. Analog is not necessarily an isomer. The above are only examples and are not limiting.

15   The term "nucleic acid sequence," "gene," "nucleic acid" or "nucleic acid cassette" as used herein refers to the genetic material of interest which can express a protein, or a peptide, or RNA after it is incorporated transiently, permanently, or episomally into a cell. The

20 nucleic acid can be positionally and sequentially oriented in a vector with other necessary elements such that the nucleic acid can be transcribed and, when necessary, translated into protein in the cells.

    The term "genetic material" as used herein refers to

25 contiguous fragments of DNA or RNA. The genetic material which is introduced into targeted cells can be any DNA or RNA. For example, the nucleic acid can be: (1) normally found in the targeted cells, (2) normally found in targeted cells but not expressed at physiologically

appropriate levels in targeted cells, (3) normally found in targeted cells but not expressed at optimal levels in certain pathological conditions, (4) not normally found in the targeted cells, (5) novel fragments of genes normally

5   expressed or not expressed in targeted cells, (6) synthetic modifications of genes expressed or not expressed within targeted cells, (7) any other DNA which may be modified for expression in targeted cells and (8) any combination of the above.

10   The term "gene expression" or "nucleic acid expression" as used herein refers to the gene product of the genetic material from the transcription and translation process. Expression includes the polypeptide chain translated from an mRNA molecule which is

15   transcribed from a gene. If the RNA transcript is not translated, e.g., rRNA, tRNA, the RNA molecule represents the gene product.

        The expression of the glucocorticoid-progesterone fusion protein receptor can be expressed as a cell

20   surface, cytoplasmic or nuclear protein. By "cell surface protein" it is meant that a protein is wholly or partially spanning the cell membrane when expressed and which also is exposed on the surface of the cell. By cytoplasmic protein it is meant that a protein is contained completely

25   within the cytoplasm, and does not span the nucleus or cell surfaces. As for "nuclear protein" it is meant that the protein is wholly or partially spanning the nuclear membrane when expressed and is exposed to the cell cytoplasm, or may be contained completely within the cell

nucleus, not attached to the nuclear membrane and not exposed to cell cytoplasm.

In a preferred embodiment, the modified glucocorticoid receptor protein includes a mutated
5   progesterone ligand binding region of amino acids 640 through 914 of a progesterone receptor ligand binding domain. In another preferred embodiment, the modified glucocorticoid receptor protein contains a transregulatory domain located in the N-terminal region of the mutated
10 progesterone ligand binding domain. In another preferred embodiment, the modified glucocorticoid receptor protein includes a transregulatory domain located in the C-terminal region of the mutated progesterone ligand binding domain. Thus, the transregulatory domain can be located
15 either in the C-terminal or N-terminal direction of the ligand binding domain.

In another preferred embodiment, the modified glucocorticoid receptor protein includes a GAL4 DNA binding domain. In another preferred embodiment, the
20 modified glucocorticoid receptor protein includes a Krüppel-associated box-A (KRAB) transrepressing domain. The terms "GAL4 DNA binding domain" and "KRAB transrepressing domain" are used as conventionally understood in the art and encompass functional equivalents
25 of such sequences that retain the ability to bind DNA or retain the transrepressing activity.

In another preferred embodiment, the modified glucocorticoid receptor protein includes a mutated progesterone receptor ligand binding region capable of

binding RU486 at a concentration as low as 0.01 nM. In still another preferred embodiment, a modified steroid hormone receptor protein responds to a conventional antagonist of the wild-type steroid hormone receptor

5   protein counterpart with an agonistic response. Those skilled in the art will understand that "binding" can be measured by several conventional methods in the art, such as binding constants and that a protein "response" can also be measured using conventional techniques in the art,

10 such as measurement of induced transcription levels.

A second aspect of the present invention features a modified glucocorticoid receptor protein. The glucocorticoid receptor protein contains a DNA binding domain, transregulatory domains and a mutated ligand

15 binding domain. The modified protein is capable of binding a non-natural ligand by the mutated ligand binding domain. The mutated ligand domain is created by deleting about 2-5 carboxyl terminal amino acids from the ligand binding domain. In a preferred embodiment, the modified

20 glucocorticoid receptor protein can be mutated by deleting amino acids 762 and 763, and substituting or altering amino acids 752 and 753, of the ligand binding domain. Substituted amino acids 752 and 753 can be changed to be both alanines.

25 A third aspect of the present invention features a modified glucocorticoid receptor protein. This protein contains a DNA binding domain and transregulatory domains. The transregulatory domains are capable of constitutively transactivating or transrepressing gene expression. The

receptor protein is mutated by removing the ligand binding domain. As used herein the term "constitutively" refers to the ability to continually activate or repress gene expression without the need for a ligand.

5   In a preferred embodiment, the modified glucocorticoid receptor protein activates target gene expression. In another preferred embodiment, the target gene encodes nerve growth factor.

A fourth aspect of the present invention features a
10 modified glucocorticoid receptor protein. This protein is capable of binding a non-natural ligand. The modified receptor contains a glucocorticoid receptor region which comprises a DNA binding domain, a mutated transregulatory domain and a mutated ligand binding domain. The mutated
15 transregulatory domains are capable of transactivating gene expression but not transrepressing gene expression. Preferably the protein activates target gene expression and the target gene encodes nerve growth factor or functional equivalents thereof.

20   Examples of the mutated transregulatory domains are described in PCT Publication PCT/US96/04324, the whole of which (including drawings) is hereby incorporated by reference.

A fifth aspect of the present invention features a
25 modified glucocorticoid receptor protein which is capable of binding a non-natural ligand. The modified receptor contains a glucocorticoid receptor region which comprises a mutated DNA binding domain, transregulatory domains and a mutated ligand binding domain. The mutated DNA binding

domain prevents transactivation since DNA binding is necessary for such activation. The transregulatory domains are capable of transrepressing gene expression but not transactivating gene repression. Such activity occurs

5   upon binding of the mutated binding ligand with the non-natural ligand.

Examples of the mutated DNA binding domain are described in PCT publication PCT/US96/04324, the whole of which (including drawings) is hereby incorporated by

10 reference.

A sixth related aspect of the invention features a nucleic acid sequence encoding one of the modified glucocorticoid receptors as discussed above, including the fusion protein receptor. The nucleic acid is the genetic

15 material which can express a protein, or a peptide, or RNA after it is incorporated transiently, permanently or episomally into a cell.

A seventh related aspect of the present invention features a vector containing a nucleic acid sequence for

20 modified glucocorticoid receptors. The vectors are capable of expressing the nucleic acid transiently, permanently or episomally into a cell or tissue. In one example, the vector is a plasmid designated as pGR0403R for the constitutively active GR and pGR0385 for mutated

25 rat GR.

The term "vector" as used herein refers to a construction comprised of genetic material designed to direct transformation of a targeted cell. A vector contains multiple genetic elements positionally and

sequentially oriented with other necessary elements such
that the nucleic acid in a nucleic acid cassette can be
transcribed and when necessary translated in the
transfected cells. The term vector as used herein can

5 refer to nucleic acid, *e.g.*, DNA derived from a plasmid,
cosmid, phagemid or bacteriophage, into which one or more
fragments of nucleic acid may be inserted or cloned which
encode for particular proteins. The term "plasmid" as
used herein refers to a construction comprised of

10 extrachromosomal genetic material, usually of a circular
duplex of DNA which can replicate independently of
chromosomal DNA. The plasmid does not necessarily
replicate.

The vector can contain one or more unique restriction

15 sites, and may be capable of autonomous replication in a
defined host or organism such that the cloned sequence is
reproduced. The vector molecule can confer some well-
defined phenotype on the host organism which is either
selectable or readily detected. The vector may have a

20 linear or circular configuration. The components of a
vector can contain but is not limited to a DNA molecule
incorporating: (1) DNA; (2) a sequence encoding a
therapeutic or desired product; and (3) regulatory
elements for transcription, translation, RNA processing,

25 RNA stability, and replication.

The purpose of the vector is to provide expression of
a nucleic acid sequence in cells or tissue. Expression
includes the efficient transcription of an inserted gene
or nucleic acid sequence. Expression products may be

proteins, polypeptides, or RNA. The nucleic acid sequence can be contained in a nucleic acid cassette. Expression of the nucleic acid can be continuous, constitutive, or regulated. The vector can also be used as a prokaryotic

5   element for replication of plasmid in bacteria and selection for maintenance of plasmid in bacteria.

In the present invention the preferred vector comprises the following elements linked sequentially at an appropriate distance to allow functional expression: a

10   promoter, a 5' mRNA leader sequence, a translation initiation site, a nucleic acid cassette containing the sequence to be expressed, a 3' mRNA untranslated region, and a polyadenylation signal sequence. As used herein the term "expression vector" refers to a DNA vector that

15   contains all of the information necessary to produce a recombinant protein in a heterologous cell.

In addition, the term "vector" as used herein can also include viral vectors. A "viral vector" in this sense is one that is physically incorporated in a viral

20   particle by the inclusion of a portion of a viral genome within the vector, e.g., a packaging signal, and is not merely DNA or a located gene taken from a portion of a viral nucleic acid. Thus, while a portion of a viral genome can be present in a vector of the present

25   invention, that portion does not cause incorporation of the vector into a viral particle and thus is unable to produce an infective viral particle.

A vector as used herein can also include DNA sequence elements which enable extra-chromosomal (episomal)

replication of the DNA.   Vectors capable of episomal replication are maintained as extra-chromosomal molecules and can replicate.   These vectors are not eliminated by simple degradation but continue to be copied.   These

5   elements may be derived from a viral or mammalian genome. These provide prolonged or "persistent" expression as described below.

The term "persistent expression" as used herein refers to introduction of genes into the cell together

10   with genetic elements which enable episomal (*i.e.*, extrachromosomal) replication.   This can lead to apparently stable transformation of the cell without the integration of the novel genetic material into the chromosome of the host cell.

15   "Stable expression" as used herein relates to the integration of genetic material into chromosomes of the targeted cell where it becomes a permanent component of the genetic material in that cell.   Gene expression after stable   integration   can   permanently   alter   the

20   characteristics of the cell and its progeny arising by replication leading to stable transformation.

An eighth related aspect of the present invention features a transfected cell containing a vector which contains   nucleic   acid   sequence   for   a   modified

25   glucocorticoid receptor as discussed above.   As used herein the term "transfected" or "transfection" refers to the incorporation of foreign DNA into any cells by exposing them to such DNA.   This would include the

introduction of DNA by various delivery methods, *e.g.*, via vectors or plasmids.

Methods of transfection may include microinjection, $CaPO_4$ precipitation, liposome fusion (*e.g.*, lipofection),

5   electroporation or use of a gene gun. Those are only examples and are meant not to be limiting. The term "transfection" as used herein refers to the process of introducing DNA (*e.g.*, DNA expression vector) into a cell. Following entry into the cell, the transfected DNA may:

10   (1) recombine with the genome of the host; (2) replicate independently as an episome; or (3) be maintained as an episome without replication prior to elimination. Cells may be naturally able to uptake DNA. Particular cells which are not naturally able to take up DNA require

15   various treatments, as described above, in order to induce the transfer of DNA across the cell membrane.

A ninth related aspect of the present invention features a transformed cell with a vector containing a nucleic acid sequence for a modified glucocorticoid

20   receptor as discussed above. As used here in the term "transformed" or "transformation" refers to transient, stable or permanent changes in the characteristics (expressed phenotype) of a cell by the mechanism of gene transfer. Genetic material is introduced into a cell in a

25   form where it expresses a specific gene product or alters the expression or effects of endogenous gene products.

The term "stable" as used herein refers to the introduction of gene(s) into the chromosome of the targeted cell where it integrates and becomes a permanent

component of the genetic material in that cell. Gene expression after stable transformation can permanently alter the characteristics of the cell leading to stable transformation. An episomal transformation is a variant

5 of stable transformation in which the introduced gene is not incorporated in the host cell chromosomes but rather is replicated as an extrachromosomal element. This can lead to apparently stable transformation of the characteristics of a cell. "Transiently" as used herein

10 refers to the introduction of a gene into a cell to express the nucleic acid, *e.g.*, the cell express specific proteins, peptides or RNA, etc. The introduced gene is not integrated into the host cell genome and is accordingly eliminated from the cell over a period of

15 time. Transient expression relates to the expression of a gene product during a period of transient transfection. Transient expression also refers to transfected cells with a limited life span.

Transformation can be performed by *in vivo* techniques

20 or *ex vivo* techniques as described in PCT Publication PCT/US96/04324, the whole of which (including drawings) is hereby incorporated by reference. Transformation can be tissue specific to regulate expression of the nucleic acid predominantly in the tissue or cell of choice.

25 Transformation of the cell may be associated with production of a variety of gene products including protein and RNA. Such products are described in PCT Publication PCT/US96/04324, the whole of which (including drawings) is hereby incorporated by reference. The product expressed

by the transformed cell depends on the nucleic acid of the nucleic acid cassette.   In the present invention the nucleic acid to be expressed is a fusion protein as referenced above, or variations thereof or any of the

5   other receptor proteins disclosed herein.

In one embodiment the transformed cell is a muscle cell.   The term "muscle" refers to myogenic cells including myoblasts, skeletal, heart and smooth muscle cells.   The muscle cells or tissue can be *in vivo*, in

10 vitro or tissue culture and capable of differentiating into muscle tissue.   In another embodiment, the transformed cell is a lung cell.   The term "lung cell" as used herein refers to cells associated with the pulmonary system.   The lung cell can also be in vivo, in vitro or

15 tissue culture.

In still another embodiment, the transformed cell is a cell associated with the joints.   The term "cells associated with the joints" refers to all of the cellular and non-cellular materials which comprise the joint (e.g.,

20 knee or elbow) and are involved in the normal function of the joint or are present within the joint due to pathological conditions.   These include material associated with: the joint capsule such as synovial membranes, synovial fluid, synovial cells (including type

25 A cells and type B synovial cells); the cartilaginous components of the joint such as chondrocyte, extracellular matrix of cartilage; the bony structures such as bone, periosteum of bone, periosteal cells, osteoblast, osteoclast; the immunological components such as

inflammatory cells, lymphocytes, mast cells, monocytes, eosinophil; other cells like fibroblasts; and combinations of the above. Once transformed these cells express the fusion protein. One skilled in the art will quickly

5 realize that any cell is capable of undergoing transformation and within the scope of this invention.

A tenth aspect of the present invention features methods for transforming a cell with a vector containing nucleic acid encoding for a modified glucocorticoid

10 receptor. This method includes the steps of transforming a cell in situ by contacting the cell with the vector for a sufficient amount of time to transform the cell. As discussed above, transformation can be *in vivo* or *ex vivo*. Once transformed the cell expresses the mutated

15 glucocorticoid receptor. This method includes methods of introducing and methods of incorporating the vector. "Incorporating" and "introducing" as used herein refer to uptake or transfer of the vector into a cell such that the vector can express the therapeutic gene product within a

20 cell as discussed with transformation above.

An eleventh aspect of the present invention features a method of using the modified glucocorticoid receptors discussed above. This method comprises the steps of transforming a cell with a vector containing a nucleic

25 acid encoding for the modified glucocorticoid receptor of interest. The transformed cells are able to express the mutated glucocorticoid receptor. The receptor is capable of regulating by a non-natural ligand the expression of glucocorticoid responsive genes, whether such regulation

is transactivation or transrepression. The term
"glucocorticoid responsive genes" as used herein refers to
genes whose expression is regulated by the activation of
the glucocorticoid receptor. Such regulation includes

5   both positive and negative regulation of gene expression.
This also includes GRE (glucocorticoid response element)
controlled genes.

This method of use includes methods of gene
replacement using the fusion protein, methods of gene

10 therapy using the fusion protein and methods of
administering the fusion protein in which the same steps
are used. "Gene replacement" as used herein means
supplying a nucleic acid sequence which is capable of
being expressed in vivo in an animal and thereby providing

15 or augmenting the function of an endogenous gene which is
missing or defective in the animal.

The methods of use also include methods for using the
modified glucocorticoid receptor to activate GRE
controlled genes. Such genes can be co-transfected with

20 the modified glucocorticoid receptors. Such co-
transfection allows activated expression of the GRE
controlled genes. Furthermore, the methods of use include
the use of tissue specific delivery systems, and use of
mRNA stability constructs.

25 The present invention features methods for
administration as discussed above. Such methods include
methods for administering a supply of polypeptide, protein
or RNA to a human, animal or to tissue culture or cells.
These methods of use of the above-referenced vectors

comprises the steps of administering an effective amount
of the vectors to a human, animal or tissue culture. The
term "administering" or "administration" as used herein
refers to the route of introduction of a vector or carrier
5   of DNA into the body. The vectors of the above methods
and the methods discussed below may be administered by
various routes.   Administration may be   intravenous,
intratissue injection, topical, oral, or by gene gun or
hypospray instrumentation. Administration can be directly
10   to a target tissue, e.g. direct injection into synovial
cavity or cells, or through systemic delivery. These are
only examples and are nonlimiting.

Administration will include a variety of methods, as
described in PCT Publication PCT/US96/04324, the whole of
15   which (including drawings) is hereby incorporated by
reference. See, also, WO 93/18759, the whole of which is
hereby   incorporated   by   reference.   The   preferred
embodiment   is   by   direct   injection.   Routes   of
administration   include   intramuscular,   aerosol,   oral,
20   topical, systemic, ocular, intraperitoneal, intrathecal
and/or fluid spaces.

The term "effective amount" as used herein refers to
sufficient vector administered to humans, animals or into
tissue culture cells to produce the adequate levels of
25   polypeptide, protein, or RNA. One skilled in the art
recognizes that the adequate level of protein polypeptide
or RNA will depend on the intended use of the particular
vector. These levels will be different depending on the

type of administration, treatment or vaccination as well as intended use.

In one embodiment of the present invention, the method of using the mutated glucocorticoid receptors

5   discussed above uses RU486 as the non-natural ligand to regulate gene expression. This ligand is capable of binding the mutated progesterone or glucocorticoid ligand binding domain and activating the transregulatory domains of the receptor. RU486 is capable of activating or

10   repressing the appropriate glucocorticoid responsive genes. This is only an example and is not meant to be limiting. Those skilled in the art will recognize that other non-natural ligands can be used.

The method of use can regulate transactivation of

15   glucocorticoid responsive genes or GRE controlled genes or gene constructs. In addition, the method of use can regulate transrepression of glucocorticoid responsive genes   such   as   metalloproteinases,   interleukins, cyclooxygenases, and cytokines. Although such genes

20   respond to other stimuli, these genes are repressed by steroids. Typically, without the primary stimulant, steroids have little effect on the basal transcription of such genes. Genes such as IL-2, IL-6, IL-8, ICAM-1, VCAM-1 have been repressed by steroids. Any gene

25   transcription depending on AP-1 or NF_κ-B will be repressed in the present invention.

A twelfth aspect of the present invention features a method for treating arthritis. This method includes the transformation of cells associated with the joints with

the above referenced vectors. The vectors contain nucleic acid which encode for the modified glucocorticoid receptor protein. Once expressed in the cells associated with the joints, the mutated protein is capable of transactivating

5   or transrepressing by a non-natural ligand the expression of glucocorticoid responsive genes or GRE controlled genes, including transfected GRE controlled gene constructs. Treatment of arthritis is further described in PCT Publication PCT/US96/04324, the whole of which

10   (including drawings) is hereby incorporated by reference.

A thirteenth aspect of the present invention features a method for treating asthma. This method includes the transformation of cells associated with the lungs or pulmonary system with the above referenced vectors. The

15   vectors contain nucleic acid which encodes the fusion protein. Once expressed in the lung cells the mutated receptor is capable of transactivating or transrepressing the expression by a non-natural ligand of the appropriate glucocorticoid responsive genes and/or GRE controlled

20   transgenes.

In one embodiment, the above methods of treatment invoke use of RU486 as the non-natural ligand. The transactivation and transrepression can occur when the mutated glucocorticoid receptor is activated by RU486. The

25   genes that are transrepressed or transactivated in response to ligand binding to the fusion protein are described above.

A fourteenth aspect of the present invention features a transgenic animal whose cells contain the vectors of the

present invention.   These cells include germ or somatic
cells.   Transgenic animal models can be used for
understanding of molecular carcinogenesis and disease,
assessing and exploring novel therapeutic avenues for
5 effects by potential chemical and physical carcinogens and
tumor promoters.

An additional preferred embodiment provides for a
transgenic animal containing a modified glucocorticoid
receptor vector.   By "transgenic animal" is meant an
10 animal whose genome contains an additional copy or copies
of the gene from the same species or it contains the gene
or genes of another species, such as a gene encoding for
a mutated glucocorticoid receptor introduced by genetic
manipulation or cloning techniques, as described herein
15 and as known in the art.   The transgenic animal can
include the resulting animal in which the vector has been
inserted into the embryo from which the animal developed
or any progeny of that animal.   The term "progeny" as used
herein includes direct progeny of the transgenic animal as
20 well as any progeny of succeeding progeny.   Thus, one
skilled in the art will readily recognize that if two
different transgenic animals have been made each utilizing
a different gene or genes and they are mated, the
possibility exists that some of the resulting progeny will
25 contain two or more introduced genes.   One skilled in the
art will readily recognize that by controlling the
matings, transgenic animals containing multiple introduced
genes can be made.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims.

Brief Description of the Drawings

5   Figure 1 shows the mutagenesis and screening strategy used in the present experiments.

Figure 2 illustrates the functional and structural characterization of the UP-1 mutant.

Figure 3 shows a western analysis of the mutant human
10   progesterone receptor.

Figure 4 shows the transcriptional activity and hormone binding analysis of wild type and mutant human progesterone receptor constructs.

Figure 5 shows the specificity of transcriptional
15   activity of the mutant human progesterone receptor.

Figure 6 depicts the transient transfection of mutant human progesterone human receptor into mammalian cells.

Figure 7 depicts the GR-PR fusion constructs.

Figure 8 depicts the Rat and Human GR double point
20   mutation constructs.

Figure 9 illustrates the nucleic acid sequence encoding a plasmid pGR0403R expressing a constitutively active mutant GR protein.

Figure 10 depicts plasmid pGR0403R expressing a
25   constitutively active mutant GR protein.

Figure 11 illustrates the amount of CAT protein produced in response to ligand binding to mutant human and rat GR and the respective wild type receptors.

Figure 12 is a schematic representation of the fusion protein with an activation transregulatory domain.

Figure 13 is a schematic representation of the gene switch.

5   Figure 14 is a schematic representation of GLVP and its derivatives containing an additional transactivation domain.

Figure 15 is a schematic representation of the effect of various lengths of poly-Q insertion on GLVP

10   transactivation potential.

Figure 16 is a schematic representation that an additional copy of the VP16 activation domain into GLVP does not further increase its transactivation potential.

Figure 17 is a diagram of the original chimeric GLVP

15   and its C-terminally extended derivatives.

Figure 18 is a diagram of the transcriptional activation of GLVP versus its C-terminally located VP16 activation domain and various extensions of the hPR-LBD.

Figure 19 is a diagram of the inducible repressors

20   and reporters constructs.

The drawings are not necessarily to scale. Certain features of the invention may be exaggerated in scale or shown in schematic form in the interest of clarity and conciseness.

25   **Detailed Description of the Invention**

The present invention provides modified proteins of steroid hormone receptors. Steroid hormone receptors which may be modified include any of those receptors which

comprise the steroid hormone receptor superfamily. Representative examples of such receptors include the estrogen, progesterone, glucocorticoid, mineralocorticoid, androgen, thyroid hormone, retinoic acid, retinoid X and

5 Vitamin D3 receptors.

The modified steroid hormone receptor proteins of the present invention include a steroid receptor region made up of a DNA binding domain, one or more transregulatory domains and a mutated steroid receptor ligand binding

10 region capable of binding a non-natural ligand.

The DNA binding domain contains the receptor regulating sequence and binds DNA. Such a domain may be a yeast GAL4 DNA binding domain. The ligand binding domain binds the specific compound which will activate the

15 receptor, for example RU486.

Several different functional domains have been characterized in transcription factors; they can be either acidic (VP16, GAL4), glutamine-rich (SP1, Oct-1, Oct2A), proline-rich (Oct3/4), or serine- and threonine-rich

20 (Pit1) (Wegner et al., *Curr. Opin. in Cell Biol.* 5:488-498 (1993)). It is known that different types of transcriptional activation domains interact with different coactivators of the general transcriptional machinery. When different activation domains are fused together in a

25 transactivator, they can synergize with each other to increase its transcriptional potential. Recently, Gerber, *et al.* demonstrated that insertion of either a poly-glutamine (poly-Q) or poly-proline (poly-P) stretch within

the GAL4-VP16 enhances the activation of GAL4-VP16 (Gerber et al., *Science* 263:808-811 (1994)).

In order to increase the potency of the GLVP regulator, varying lengths of poly-Q stretches encoded by the triplet repeats (CAG)n were inserted into the N-terminus of the GLVP regulator (Figure 14). Transactivation analysis of the various sizes of poly-Q insertions in the GLVP indicate that addition of 10-34Q increases transcriptional activity of the regulator on the reporter gene (17x4-TATA-hGH), while further extension of poly-Q from a 66Q-oligomer to a 132Q-oligomer results in decreased activation of target gene (Figure 15). These experiments demonstrated that a combination of different types of functional domains of appropriate strength further improves the activation potential of the GLVP chimeric regulator.

To understand whether additional activation domains of the same type would also increase the activation potential of the chimeric regulator, GLVPx2 with 2 copies of VP16 activation domain at the N-terminus was constructed (Figure 14). As shown in Figure 16, further addition of the same type of transactivation domain (VP16) did not increase the activation potential of the regulator.

The original GLVP can efficiently activate target gene expression containing stronger promoters such as the thymidine kinase (tk) promoter. To further enhance the transcriptional activity of GLVP, a more potent RU486-inducible gene regulator was generated. This new gene

regulator responds to RU486 at a concentration even lower than that used by the original GLVP. At this concentration, RU486 does not have any anti-progesterone or anti-glucocorticoid activity. The inducible system has been used successfully to produce secreted NGF from a reporter gene in an RU486 dependent manner to induce neurite outgrowth in co-cultured PC12 cells (of rat adrenal pheochromocytoma). This RU486-controllable ligand binding domain can also be converted to an inducible repressor for shutting down target gene expression. Individual domains within a chimeric fusion protein have been shown to influence each other's function in a position-dependent context.

Transcriptional regulation of gene expression has been intensively studied over the past decade (McKnight, *Genes Dev.* 10:367-381; Goodrich et al., *Curr. Opin. in Cell Biol.* 6:403-409; Pugh, *Curr. Opin. in Cell Biol.* 8:303-311). It is generally believed that transcription factors selectively bind to their recognition sequences on DNA (promoters and enhancers) and directly interact with the TBP-associated factors (TAFs), coactivators, or corepressors to activate or repress transcriptional activity. Nuclear hormone receptors, such as steroid, thyroid, retinoid and orphan receptors, are an unique class of inducible transcription factors that can modulate their respective target genes in response to their cognate ligands. Recently, several coactivators (SRC-1) (Onate et al., *Science* 270:1354-1357 (1995)), CBP (Kamei et al., *Cell* 85:403-414 (1996)), and corepressors (N-CoR, SMART)

(Hörlein et al., *Nature* 377:397-404 (1995); Chen et al., *Nature* 377:454-457 (1995)), that mediate nuclear hormone receptor activation of target genes have been identified. These studies suggest that multiple protein factors are

5   involved in the complex process of transcriptional regulation of gene expression.

Mutagenesis studies of the hPR ligand binding domain have demonstrated that extension of the LBD deletion from amino acid position 891 to 914 increases the activation

10 potential of the chimeric regulator. Addition of this short stretch of 23 amino acids increases the PR-LBD's dimerization potential and subsequent binding to its response element. Further extension of the hPR-LBD from residue 917 to 928 results in a decrease of

15 transactivation, suggesting that this region may serve as a repressor interacting domain. In fact, when this 12 amino acid stretch is ligated to the GAL4 DNA binding domain, it is sufficient to confer transcriptional repression of a target gene, suggesting that these 12

20 amino acids might interact with a yet unidentified cellular co-repressor (Xu et al., (unpublished) (1996)).

Many chimeric proteins have been constructed in recent years in order to combine different functional domains of various proteins into one versatile chimera.

25 While it is clear that each protein domain can function independently, relatively little is known about how individual domains modulate each other's function within a chimeric protein. The activation potential of VP16 is influenced by its relative position within the chimeric

regulator. The C-terminally located VP16 chimeric regulator $GL_{914}VP_{C'}$ effectively activates target gene expression containing a minimal promoter at an RU486 concentration 10-fold lower than its N-terminally located

5   VP16 counterpart, $GL_{914}VP$. At this concentration, RU486 is expected to have no interference with endogenous gene expression.

This new inducible system will afford an improved margin of safety and further contribute to its application

10   for gene regulation *in vivo*. Mutational studies revealed that the chimeric regulator $GL_{914}VP_{C'}$ is about 8 to 10 times more potent than our originally described regulator GLVP and responds at a lower ligand concentration. Furthermore, within a chimeric protein, individual

15   functional domains, such as those involved in transactivation, DNA binding and ligand binding, can modulate each other's function, depending on their relative positions.

Protein-protein interaction studies suggest that

20   different types of transactivation or transrepression domains interact with their respective TAFs or coactivator, corepressor molecules within the RNA polymerase II preinitiation complex to alter gene transcription (Pugh, *Curr. Opin. in Cell Biol.* 8:303-311;

25   Goodrich et al., *Cell* 75:519-530 (1993)). Glutamine rich stretches have been identified in various transcriptional factors (SP1, Oct-1 and androgen receptor) although their precise function is unknown (Wegner et al., *Curr. Opin. in Cell Biol.* 5:488-498 (1993); Gerber et al., *Science*

263:808-811 (1994)). Expanded regions of triplet CAG
repeats have been implicated in several neurodegenerative
diseases such as Huntington's, Kennedy's, dentatorubral-
pallidoluysian atrophy (DRPLA), and hereditary
5   spinocerebellar ataxias (SCA1) (Kuhl et al., *Curr. Opin.
in Genet. Dev.* 3:404-407 (1993); Ross et al., *Trends in
Neurosci* 16:254-260 (1993)); Ashley et al., *Annu. Rev.
Genet.* 29:703-728 (1995)).

Recently, several groups have isolated proteins
10 responsible for the above mentioned neurodegenerative
diseases and confirmed that they indeed contain long poly-
glutamine (Q) stretches encoded by the expanded CAG
repeats (Servadio et al., *Nature Genet.* 10:94-98 (1995);
Yazawa et al., *Nature Genet.* 10:99-103 (1995); Trottier et
15 al., *Nature Genet.* 10:104-110 (1995)). To further
understand the role of poly-Q stretches in transcriptional
regulation, various lengths of poly-Q was inserted in the
N-terminus of GLVP.

Addition of a 10-34 oligomer of poly-Q results in
20 synergistic transcriptional activation, while expanded CAG
triplet repeats beyond 66 oligomeric glutamines do not
further increase the transactivation potential of chimeric
regulator GLVP. These observations suggest that
structural and conformational changes might be involved in
25 proteins encoded by the expanded CAG triplet repeat as
compared with the regular length poly-Q which encoded by
10-30 repeats of CAG in normal protein. These results
suggest that a neurological disease with expanded CAG
repeats (>40 mer) may not be due to aberrant high

transcriptional potential but rather due to an influence on other aspects of cell function (Burke et al., *Nature Medecine* 2:347-350 (1996)).

5   A transcription factor can either activate or repress gene expression depending on the promoter/enhancer context of its particular target DNA and the coregulator proteins with which it interacts (Kingston et al. *Genes Dev.* 10:905-920 (1996)).   For example, in the absence of thyroid hormone (T3), the thyroid hormone receptor (TR)

10   normally binds to its recognition sequence on DNA and represses target gene activation through interactions with corepressors (Baniahmad et al., *Mol. Cell. Biol.* 15:76-86 (1995); Shibata et al. (unpublished) (1996); Chen et al., *Nature* 377:454-457 (1995)).   In the presence of T3, the

15   co-repressor is released from the receptor and coactivators are recruited to enhance gene expression. Many transcription factors, such as p53, WT-1, YY1, Rel, can also act as dual activators and repressors depending on the DNA template and protein co-factors with which they

20   interact.

The *Drosophila* zinc finger transcription factor, Krüppel, is encoded by a gap gene and is essential for organogenesis during later stages of the development. Through *in vitro* protein-protein interaction studies,

25   Sauer et al. have demonstrated that the Krüppel protein can act as a transcriptional activator at low protein concentration (monomeric form) by interacting with TFIIB. However, at higher protein concentration, Krüppel forms a dimer and directly interacts with TFIIEβ resulting in

transcriptional repression. Several Krüppel related
proteins recently have been identified in mammalian cells
(Witzgall et al., *Mol. Cell. Biol.* 13:1933-42 (1993);
Witzgall et al., *Proc. Natl. Acad. Sci.* 91:4514-4518
5   (1994); Margolin et al., *Proc. Natl. Acad. Sci.* 91:4509-
4513 (1994)). One of them, *Kid-1*, was isolated from rat
kidney and contains a highly conserved region of ~75 amino
acids at the N-terminus termed Krüppel-associated box
(KRAB).

10   It has been shown that the KRAB domain can act as a
potent repressor when fused to a yeast GAL4 DNA binding
domain or TetR (Deuschle et al., *Mol. Cell. Biol.* 15:1907-
1914 (1995)). Replacement of the VP16 transcriptional
activation domain with the *Kid-1* KRAB repression domain,
15   converted a regulatable transactivator into a regulatable
repressor. By exchanging the GAL4 DNA binding domain with
the DNA binding domain of another protein, repression of
a target gene (e.g., tumor proliferation gene) may be
achieved in response to ligand RU486. Recently, Deuschle
20   *et al.* reported that the KRAB domain isolated from *Kox1*
zinc finger protein, which shares extensively homology
with that of *Kid-1*, interacts with a 110 kDa adaptor
protein termed SMP1 (silencing-mediating protein 1). The
characteristics and mechanism of this adaptor protein have
25   yet to be determined. Recently, a KRAB-associated
protein-1 (KAP-1) was identified which binds to the KRAB
domain and functions as a transcriptional co-repressor
(Friedman et al., *Gene and Dev.* 10:2067 (1996)).

Using the newly modified $GL_{914}VP_{C'}$, regulation of neurite outgrowth in PC12 cells via RU486 controllable expression of NGF was achieved. This novel inducible system can be employed to analyze biological function in

5   a temporal manner. For example, the role of a growth factor could be assessed at a particular stage of development and the sequential relationship of *in vivo* cell death and proliferation could be delineated in a manner not possible with constitutive expression of the

10   test gene.

Tissue specific regulation of gene expression in transgenic mice utilizing this inducible system was demonstrated. RU486 inducible regulator may be used to create an inducible gene knockout (temporal and/or

15   spatial) in transgenic mice which could circumvent an embryonic lethality resulting from use of current gene knockout techniques. Combinatorial inclusion of other inducible systems such as the tetracycline or ecdysone system with the RU486 inducible system may allow

20   biologists one day to modulate complex biological processes which involve multiple levels of control.

The following are examples of the present invention using the mutated steroid receptors for gene therapy. It will be readily apparent to one skilled in the art that

25   various substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. Thus, these examples are offered by way of illustration and are not intended to limit the invention in any manner.

The following are specific examples of preferred embodiments of the present invention. These examples demonstrate how the molecular switch mechanisms of the present invention can be used in construction of various cellular or animal models and how such molecular switch mechanisms can be used to transactivate or transrepress the regulation of gene expression. The utility of the molecular switch molecules is noted herein and is amplified upon in related applications by O'Malley et al, entitled "Modified Steroid Hormones for Gene Therapy and Methods for Their Use," and by Vegeto, et al., entitled "Mutated Steroid Hormone Receptors, Methods for Their Use and Molecular Switch for Gene Therapy," *supra* and in a related U.S. Patent by Vegeto, et al., entitled "Progesterone Receptor Having C-Terminal Hormone Binding Domain Truncations," *supra*. Such sections (including drawings) are hereby specifically incorporated by reference herein.

Methods of Use

Cell Transformation

One embodiment of the present invention includes cells transformed with nucleic acid encoding for the mutated receptor. Once the cells are transformed, the cells will express the protein, polypeptide, or RNA encoded for by the nucleic acid. Cells include but are not limited to joints, lungs, muscle and skin. This is not intended to be limiting in any manner.

The nucleic acid which contains the genetic material of interest is positionally and sequentially oriented within the host or vectors such that the nucleic acid can be transcribed into RNA and, when necessary, be translated

5   into proteins or polypeptides in the transformed cells. A variety of mutated glucocorticoid proteins and polypeptides can be expressed by the sequence in the nucleic acid cassette in the transformed cells.

Transformation can be done either by *in vivo* or *ex*

10 *vivo* techniques. One skilled in the art will be familiar with such techniques for transformation. Transformation by *ex vivo* techniques includes co-transfecting the cells with DNA containing a selectable marker. This selectable marker is used to select those cells which have become

15 transformed. Selectable markers are well known to those who are skilled in the art.

For example, one approach to gene therapy for muscle diseases is to remove myoblasts from an affected individual, genetically alter them *in vitro*, and reimplant

20 them into a receptive locus. The *ex vivo* approach includes the steps of harvesting myoblasts cultivating the myoblasts, transducing or transfecting the myoblasts, and introducing the transfected myoblasts into the affected individual.

25 The myoblasts may be obtained in a variety of ways. They may be taken from the individual who is to be later injected with the myoblasts that have been transformed or they can be collected from other sources, transformed and then injected into the individual of interest.

Once the *ex vivo* myoblasts are collected, they may be transformed by contacting the myoblasts with media containing the nucleic acid transporter and maintaining the cultured myoblasts in the media for sufficient time and
5   under conditions appropriate for uptake and transformation of the myoblasts. The myoblasts may then be introduced into an appropriate location by injection of cell suspensions into tissues. One skilled in the art will recognize that the cell suspension may contain: salts,
10   buffers or nutrients to maintain viability of the cells; proteins to ensure cell stability; and factors to promote angiogenesis and growth of the implanted cells.

In an alternative method, harvested myoblasts may be grown *ex vivo* on a matrix consisting of plastics, fibers
15   or gelatinous materials which may be surgically implanted in an appropriate location after transduction. This matrix may be impregnated with factors to promote angiogenesis and growth of the implanted cells. Cells can then be reimplanted.

20   <u>Administration</u>

Administration as used herein refers to the route of introduction of a vector or carrier of DNA into the body. Administration may include intravenous, intramuscular, topical, or oral methods of delivery. Administration can
25   be directly to a target tissue or through systemic delivery.

In particular, the present invention can be used for treating disease or for administering the formulated DNA

expression vectors capable of expressing any specific nucleic acid sequence. Administration can also include administering a regulatable vector discussed above. Such administration of a vector can be used to treat disease.

5   The preferred embodiment is by direct injection to the target tissue or systemic administration.

A second critical step is the delivery of the DNA vector to the nucleus of the target cell where it can express a gene product. In the present invention this is

10 accomplished by formulation. The formulation can consist of purified DNA vectors or DNA vectors associated with other formulation elements such as lipids, proteins, carbohydrates, synthetic organic or inorganic compounds. Examples of such formulation elements include, but are not

15 limited to, lipids capable of forming liposomes, cationic lipids, hydrophilic polymers, polycations (e.g., protamine, polybrene, spermidine, polylysine), peptide or synthetic ligands recognizing receptors on the surface of the target cells, peptide or synthetic ligands capable of

20 inducing endosomal lysis, peptide or synthetic ligands capable of targeting materials to the nucleus, gels, slow release matrices, soluble or insoluble particles, as well as other formulation elements not listed. This includes formulation elements for enhancing the delivery, uptake,

25 stability, and/or expression of genetic material into cells.

The delivery and formulation of any selected vector construct will depend on the particular use for the expression vectors. In general, a specific formulation

for each vector construct used will focus on vector uptake with regard to the particular targeted tissue, followed by demonstration of efficacy. Uptake studies will include uptake assays to evaluate cellular uptake of the vectors

5   and expression of the tissue specific DNA of choice. Such assays will also determine the localization of the target DNA after uptake, and establish the requirements for maintenance of steady-state concentrations of expressed protein. Efficacy and cytotoxicity can then be tested.

10 Toxicity will not only include cell viability but also cell function.

DNA uptake by cells associated with fluid spaces have the unique ability to take up DNA from the extracellular space after simple injection of purified DNA preparations

15 into the fluid spaces. Expression of DNA by this method can be sustained for several months.

Incorporating DNA by formulation into particulate complexes of nanometer size that undergo endocytosis increases the range of cell types that will take up

20 foreign genes from the extracellular space.

Formulation can also involve DNA transporters which are capable of forming a non-covalent complex with DNA and directing the transport of the DNA through the cell membrane. This may involve the sequence of steps including

25 endocytosis and enhanced endosomal release. It is preferable that the transporter also transport the DNA through the nuclear membrane. See, *e.g.*, the following applications all of which (including drawings) are hereby incorporated by reference herein: (1) Woo et al., U.S.

Serial No. 07/855,389, entitled "A DNA Transporter System
and Method of Use" filed March 20, 1992; (2) Woo et al.,
PCT/US93/02725, entitled "A DNA Transporter System and
Method of Use", (designating the U.S. and other countries)

5   filed March 19, 1993; and (3) continuation-in-part appli-
cation by Woo et al., entitled "Nucleic Acid Transporter
Systems and Methods of Use", filed December 14, 1993,
assigned U.S. Serial Number 08/167,641.

    In addition, delivery can be cell specific or tissue
10 specific by including cell or tissue specific promoters.
Furthermore, mRNA stabilizing sequences (3' UTR's) can be
used to provide stabilized modified receptor molecules.
Such stabilizing sequences increase the half-life of mRNAs
and can be cell or tissue specific.   The above is
15 discussed in more detail in U.S. Patent 5,298,422
(Schwartz et al.) and U.S. Application Serial Number
08/209,846 (Schwartz et al.), filed March 9, 1994,
entitled "Expression Vector Systems and Method of Use."
Both of these, the whole of which, are incorporated by
20 reference herein, including drawings.

    In a preferred method of administration involving a
DNA transporter system, the DNA transporter system has a
DNA binding complex with a binding molecule capable of
non-covalently binding to DNA which is covalently linked
25 to a surface ligand.   The surface ligand is capable of
binding to a cell surface receptor and stimulating entry
into the cell by endocytosis, pinocytosis, or potocytosis.
In addition, a second DNA binding complex is capable of
non-covalently binding to DNA and is covalently linked to

a nuclear ligand. The nuclear ligand is capable of recognizing and transporting a transporter system through a nuclear membrane. Additionally, a third DNA binding complex may be used which is also capable of non-

5   covalently binding to DNA. The third binding molecule is covalently linked to an element that induces endosomal lysis or enhanced release of the complex from the endosome after endocytosis. The binding molecules can be spermine, spermine derivatives, histones, cationic peptides and/or

10 polylysine. See also Szoka, C.F., Jr. et al., Bioconjug. Chem. 4:85-93 (1993); Szoka, F.C., Jr. et al., P.N.A.S., 90:893-897 (1993).

Transfer of genes directly has been very effective. Experiments show that administration by direct injection

15 of DNA into joint tissue results in expression of the gene in the area of injection. Injection of plasmids containing the mutated receptors into the spaces of the joints results in expression of the gene for prolonged periods of time. The injected DNA appears to persist in

20 an unintegrated extrachromosomal state. This means of transfer is the preferred embodiment.

The formulation used for delivery may also be by liposomes or cationic lipids. Liposomes are hollow spherical vesicles composed of lipids arranged in a

25 similar fashion as those lipids which make up the cell membrane. They have an internal aqueous space for entrapping water soluble compounds and range in size from 0.05 to several microns in diameter. Several studies have shown that liposomes can deliver nucleic acids to cells

and that the nucleic acid remains biologically active.
Cationic lipid formulations such as formulations incorpor-
ating DOTMA has been shown to deliver DNA expression
vectors to cells yielding production of the corresponding

5 protein.   Lipid formulations may be non-toxic and biode-
gradable in composition.   They display long circulation
half-lives and recognition molecules can be readily
attached to their surface for targeting to tissues.
Finally, cost effective manufacture of liposome-based

10 pharmaceuticals,   either   in   a   liquid   suspension   or
lyophilized product, has demonstrated the viability of
this technology as an acceptable drug delivery system.
See Szoka, F.C., Jr. et al., Pharm. Res., 7:824-834
(1990); Szoka, F.C., Jr. et al., Pharm. Res., 9:1235-1242

15 (1992).

The   chosen   method   of   delivery   should   result   in
nuclear or cytoplasmic accumulation and optimal dosing.
The dosage will depend upon the disease and the route of
administration but should be between 1-1000 μg/kg of body

20 weight.   This level is readily determinable by standard
methods.   It could be more or less depending on the opti-
mal dosing.   The duration of treatment will extend through
the course of the disease symptoms, possibly continuously.
The   number   of   doses   will   depend   upon   disease,   the

25 formulation and efficacy data from clinical trials.

With respect to vectors, the pharmacological dose of
a vector and the level of gene expression in the appro-
priate cell type includes but is not limited to sufficient
protein or RNA to either: (1) increase the level of

protein production; (2) decrease or stop the production of a protein; (3) inhibit the action of a protein; (4) inhibit proliferation or accumulation of specific cell types; and (5) induce proliferation or accumulation of

5 specific cell types. As an example, if a protein is being produced which causes the accumulation of inflammatory cells within the joint, the expression of this protein can be inhibited, or the action of this protein can be interfered with, altered, or changed.

10 <u>Persistent Expression Using Episomal Vectors</u>

In each of the foregoing examples, transient expression of recombinant genes induces the desired biological response. In some diseases more persistent expression of recombinant genes is desirable. This is achieved by

15 adding elements which enable extrachromosomal (episomal) replication of DNA to the structure of the vector. Vectors capable of episomal replication are maintained as extrachromosomal molecules and can replicate. These sequences will not be eliminated by simple degradation but

20 will continue to be copied. Episomal vectors provide prolonged or persistent, though not necessarily stable or permanent, expression of recombinant genes in the joint. Persistent as opposed to stable expression is desirable to enable adjustments in the pharmacological dose of the

25 recombinant gene product as the disease evolves over time.

Formulations for Gene Delivery into Cells of the Joint

Initial experiments used DNA in formulations for gene transfer into cells of the joint. This DNA is taken up by synovial cells during the process of these cells continu-

5   ally resorbing and remodeling the synovial fluid by secretion and pinocytosis. Gene delivery is enhanced by packaging DNA into particles using cationic lipids, hydrophilic (cationic) polymers, or DNA vectors condensed with polycations which enhance the entry of DNA vectors

10 into contacted cells. Formulations may further enhance entry of DNA vectors into the body of the cell by incor-porating elements capable of enhancing endosomal release such as certain surface proteins from adenovirus, influen-za virus hemagglutinin, synthetic GALA peptide, or

15 bacterial toxins. Formulations may further enhance entry of DNA vectors into the cell by incorporating elements capable of binding to receptors on the surface of cells in the joint and enhancing uptake and expression. Alternatively, particulate DNA complexed with polycations

20 can be efficient substrates for phagocytosis by monocytes or other inflammatory cells. Furthermore, particles containing DNA vectors which are capable of extravasating into the inflamed joint can be used for gene transfer into the cells of the joint. One skilled in the art will

25 recognize that the above formulations can also be used with other tissues as well.

Induction of "Steroid Response" by Gene Transfer of Steroid Receptors into Cells of the Joint

Current therapy for severe arthritis involves the administration of pharmacological agents including
5   steroids to depress the inflammatory response. Steroids can be administered systemically or locally by direct injection into the joint space.

Steroids normally function by binding to receptors within the cytoplasm of cells. Formation of the steroid-
10   receptor complex changes the structure of the receptor so that it becomes capable of translocating to the nucleus and binding to specific sequences within the genome of the cell and altering the expression of specific genes. Genetic modifications of the steroid receptor can be made
15   which enable this receptor to bind non-natural steroids. Other modifications can be made to create a mutated steroid receptor which is "constitutively active" meaning that it is capable of binding to DNA and regulating gene expression in the absence of steroid in the same way that
20   the natural steroid receptor regulates gene expression after treatment with natural or synthetic steroids.

Of particular importance is the effect of glucocorticoid steroids such as cortisone, hydrocortisone, prednisone, or dexamethasone which are effective drugs
25   available for the treatment of arthritis. One approach to treating arthritis is to introduce a vector in which the nucleic acid cassette expresses a genetically modified steroid receptor into cells of the joint, e.g., a genetically modified steroid receptor which mimics the

effect of glucocorticoid but does not require the presence of glucocorticoid for effect. This is achieved by expression of a fusion receptor protein discussed above or other mutated glucocorticoid receptors such as ones which

5 are constitutively active within cells of the joint. This induces the therapeutic effects of steroids without the systemic toxicity of these drugs.

Alternatively, construction of a steroid receptor which is activated by a novel, normally-inert steroid

10 enables the use of drugs which would affect only cells taking up this receptor. These strategies obtain a therapeutic effect from steroids on arthritis without the profound systemic complications associated with these drugs. Of particular importance is the ability to target

15 these genes differentially to specific cell types (for example synovial cells versus lymphocytes) to affect the activity of these cells.

The steroid receptor family of gene regulatory proteins is an ideal set of such molecules. These

20 proteins are ligand activated transcription factors whose ligands can range from steroids to retinoic acid, fatty acids, vitamins, thyroid hormones and other presently unidentified small molecules. These compounds bind to receptors and either activate or repress transcription.

25   The preferred receptor of the present invention is modification of the glucocorticoid receptor, i.e., the fusion protein receptor. These receptors can be modified to allow them to bind various ligands whose structure differs from naturally occurring ligands. For example,

small C-terminal alterations in amino acid sequence, including truncation, result in altered affinity of ligand binding to the progesterone receptor. By screening receptor mutants, receptors can be customized to respond
5   to ligands which do not activate the host cell endogenous receptors.

A person having ordinary skill in the art will recognize, however, that various mutations, for example, a shorter deletion of carboxy terminal amino acids, will
10 be necessary to create useful mutants of certain steroid hormone receptor proteins. Steroid hormone receptors which may be mutated are any of those receptors which comprise the steroid hormone receptor superfamily, such as receptors including the estrogen, progesterone,
15 glucocorticoid-α, glucocorticoid-β, mineral corticoid, androgen, thyroid hormone, retinoic acid, and Vitamin D3 receptors.

Direct DNA Delivery to Muscle

Diseases that result in abnormal muscle development,
20 due to many different reasons can be treated using the above modified glucocorticoid receptors. These diseases can be treated by using the direct delivery of genes encoding for the mutated glucocorticoid receptor of the present invention resulting in the production of mutated
25 receptor gene product. Genes which can be repressed or activated have been outlined in detail above.

Direct DNA Delivery to the Lungs

Current therapy for severe asthma involves the administration of pharmacological agents including steroids to inhibit the asthma response. Steroids can be administered systemically or locally by direct

5   instillation or delivery into the lungs.

Of particular importance is the effect of glucocorticoid steroids such as cortisone, hydrocortisone, prednisone, or dexamethasone which are the most important-effective drugs available for the treatment of asthma.

10   One approach to treating asthma is to introduce a vector in which the nucleic acid cassette expresses a genetically modified steroid receptor into cells of the lungs, *e.g.*, a genetically modified steroid receptor which mimics the effect of glucocorticoid but does not require the presence

15   of glucocorticoid for effect. This is achieved by expression of the fusion proteins discussed above or other mutated glucocorticoid receptors such as ones which are constitutively active within cells of the lungs. This induces the therapeutic effects of steroids without the

20   systemic toxicity of these drugs.

Alternatively, construction of a steroid receptor which is activated by a novel, normally-inert steroid enables the use of drugs which would affect only cells taking up this receptor. These strategies obtain a

25   therapeutic effect from steroids on asthma without the profound systemic complications associated with these drugs. Of particular importance is the ability to target these genes differentially to specific cell types (for

example alveoli of the lungs) to affect the activity of these cells.

The steroid receptor family of gene regulatory proteins is an ideal set of such molecules. These

5   proteins are ligand-activated transcription factors whose ligands can range from steroids to retinoids, fatty acids, vitamins, thyroid hormones, and other presently unidentified small molecules. These compounds bind to receptors and either up-regulate or down-regulate

10 transcription.

The preferred receptor of the present invention is the modified glucocorticoid receptor. These receptors can be modified to allow them to bind various ligands whose structure differs from naturally occurring ligands. For

15 example, small C-terminal alterations in amino acid sequence, including truncation, result in altered affinity of the ligand and altered function. By screening receptor mutants, receptors can be customized to respond to ligands which do not activate the host cells own receptors.

20 A person having ordinary skill in the art will recognize, however, that various mutations, for example, a shorter deletion of carboxy terminal amino acids, will be necessary to create useful mutants of certain steroid hormone receptor proteins. Steroid hormone receptors

25 which may be mutated are any of those receptors which comprise the steroid hormone receptor superfamily, such as receptors including the estrogen, progesterone, glucocorticoid-α, glucocorticoid-β, mineral corticoid,

androgen, thyroid hormone, retinoic acid, and Vitamin D3 receptors.

EXAMPLES

5   While the present invention is disclosed by reference to the details for the following examples, it is to be understood that this disclosure is intended in an illustrative rather than limiting sense, as it is contemplated that modifications will readily occur to those skilled in the art, within the spirit of the

10   invention and the scope of the appended claims.

Mutagenesis and Characterization of the Ligand Binding Domain of Human Progesterone Receptor

EXAMPLE 1

Yeast Strain

15   The *Saccharomyces cerevisiae* strain BJ3505 (MATα, pep4:HIS3, prb1-Δ1.6R, his3Δ200, lys2-801, trp1-Δ101, ura3-52, gal2, (CUP1)) was used (Yeast Genetic Stock Center, Berkeley, CA). All yeast transformations were carried out following the lithium acetate transformation

20   protocol (Ito, et al., *J. Bacteriol.* 153:163-168, 1983).

The PCR reactions were carried out using YEphPR-B DNA template (a YEp52AGSA-derived yeast expression plasmid containing the cDNA of hPR form-B (Misrahi, et al., *Biochem. Bioph. Res. Comm.* 143:740-748, 1987) inserted

25   downstream of the yeast methallothionein-CUP1 promoter) and using three different sets of primers. In order to

decrease the fidelity of the second strand polymerization reaction, buffer conditions of 1.5 mM $MgCl_2$, 0.1 mM dNTPs and pH 8.2 were used.   About 2000 primary transformants were obtained from each region-specific library.

5   EXAMPLE 2

Yeast Mutant Screening

        Colonies of each library of hPR molecules mutated in specific subregions were pooled, large amounts of DNA were prepared and used to transform yeast cells carrying the
10   reporter plasmid YRpPC3GS+, which contains two GRE/PRE elements upstream of the CYC1 promoter linked to the Lac-Z gene of *E. coli* (Mak, et al., *J. Biol. Chem.* 265:20085-20086, 1989).   The transformed cells were plated on 1.5% agar plates containing 2% glucose, 0.5% casamino acids (5%
15   stock solution of casamino acids is always autoclaved before use to destroy tryptophan), 6.7 g/l yeast nitrogen base (without amino acids) and 100 μM CuSO4 (CAA/Cu plates) and grown for 2 days at 30°C.   These colonies were then replica-plated on CAA/Cu plates containing 0.16 g/l
20   of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal, an indicator of β-galactosidase activity) with or without the hormones as indicated in Fig. 1 and allowed to grow for one day at 30°C, then two days at room temperature in the dark.

25   EXAMPLE 3

Growth of Yeast Culture for *In Vitro* Assay

Saccharomyces cerevisiae cells containing YEphPRB and
the reporter plasmid were grown overnight at 30°C in
minimal media containing 2% glucose.   The cells were
subcultured in fresh medium and allowed to grow until
5   early mid-log phase (O.D.$_{600nm}$=1.0).   Induction of receptor
was initiated by the addition of 100 μM copper sulfate to
the culture.   Cells were harvested by centrifugation at
1,500 xg for 10 minutes and resuspended in the appropriate
buffer.   This and all subsequent steps of analysis of the
10   yeast extracts were done at 4°C.

EXAMPLE 4

Transcription Assay

Yeast cells containing the reporter and expression
plasmids were grown overnight as described above in
15   Example 3 in the presence of 100 μM copper sulfate.   When
the cell density reached O.D.$_{600nm}$=1.0, hormones were added
to the cultures.   After a 4 hour incubation, yeast ex-
tracts were prepared and assayed for β-galactosidase
activity as described previously (Miller, J. M. Miller
20   ed., 352-355 (1972)).

Generally, reporters useful in the present invention
are any which allow for appropriate measurement of tran-
scription levels.   Preferable reporter systems include
reporter vectors comprised of the yeast iso-1-cytochrome
25   C proximal promoter element fused to a structural gene,
wherein said structural gene is selected from the group
consisting of β-galactosidase, galactokinase and URA3.
More preferably, the vector is comprised of an insertion

site for a receptor response element. The vectors which include β-galactokinase as an indicator of transcriptional activity are derived from the parent vector PC2 while the vectors which include galactokinase are derived from YCpR1

5   vector. Preferably, the structural genes originate from *E. coli*.

EXAMPLE 5

Western Immunoblotting

Yeast cells were grown as discussed above for the

10 transcription assay. Yeast extracts for Western blot analysis were prepared by resuspending the cell pellet in TEDG+salts. The cell suspension was mixed with an equal volume of glass beads and disrupted by vortexing in a microcentrifuge tube. The homogenate was centrifuged at

15 12,000 x g for 10 minutes. The supernatant was collected and the protein concentration was estimated using bovine serum albumin as standard. Yeast extracts were resolved on a 0.1% sodium dodecyl sulfate-7% polyacrylamide gel and transferred to Immobilon membrane as described previously

20 (McDonnell, et al., *Mol. Cell. Biol.* 9:3517-3523, 1989). Solid phase radioimmunoassay was performed using a mono-clonal antibody (JZB39) directed against the N-terminal domain of A and B forms of hPR.

EXAMPLE 6

25 Hormone Binding Competition Assays

Induction of PR synthesis was initiated by the addi-tion of 100 μM $CuSO_4$ to the culture and incubation was

continued for 6 hours. The cell pellet was resuspended in
TESH buffer containing 1 μg/ml leupeptin, 10 μg/ml PMSF
and 10 μg/ml pepstatin. The cell suspension was mixed
with an equal volume of glass beads (0.5 mm; B. Braun
5 Instruments) and disrupted by vortexing in a micro-
centrifuge tube. The homogenate was centrifuged at 12,000
x g for 10 minutes and the supernatant was further centri-
fuged at 100,000 x g for 30 minutes to obtain a cytosol
fraction. Diluted yeast extracts (200 μl) containing 100
10 μg of total protein were incubated overnight at 4°C with
[^{3}H]ligand in the absence (total binding) or presence (non-
specific binding) of a 100-fold excess of unlabeled
ligand. Bound and free steroids were separated by addi-
tion of 500 μl of dextran-coated charcoal suspension (0.5%
15 Norit A, 0.05% dextran, 10 mM Tris HCl, pH 7.4 and 1 mM
EDTA). Specific binding was determined by subtracting
nonspecific from total binding. Scatchard analysis was
carried out as described previously by Mak, et al., *J.*
Biol. Chem. 264:21613:21618 (1989).

20   EXAMPLE 7

Site-directed Mutagenesis

       Mutants YEphPR-B879 and YEphPR-B891 were prepared
following the procedure described by Dobson, et al., *J.*
Biol. Chem. 264:4207-4211 (1989). CJ236 cells were
25 infected with mpPR90 (an M13 plasmid containing hPR cDNA).
The resulting uridine-containing single-stranded DNA was
annealed to 20-mer oligonucleotides containing a TGA stop

codon corresponding to amino acids 880 and 892, respectively.

EXAMPLE 8

Construction of Mammalian Expression Vectors

The mammalian expression vector phPR-B contains the

5   SV40 enhancer sequence upstream of the human growth

hormone promoter linked to the hPR-B cDNA. This vector

was digested with Sall and EcoRl. The 6.1kb fragment

(containing the vector sequences and the 5'-1.5 kb of the

hPR) was gel-purified and ligated to the 2.1 kb fragment

10 of YEphPR-B891 (containing the 3'-end of the receptor)

previously cut with Sall and EcoRl. The resulting plas-

mid, phPR-B891, encodes a 42 amino acid truncated version

of hPR form B.

EXAMPLE 9

15 Mammalian Cell Transient Transfections and CAT-Assays

Five micrograms of chloramphenicol acetyltransferase

(CAT) reporter plasmid, containing two copies of a PRE/GRE

from the tyrosine amino transferase gene linked to the

thymidine kinase promoter (PRETKCAT), were used in

20 transient cotransfection experiments together with 5 μg of

wild type or mutant receptor DNAs.   Transient

cotransfections and CAT-assays were performed as described

by Tsai et al., *Cell* 57:443-448 (1989).

EXAMPLE 10

25 Mutagenesis of the Hormone Binding Domain of hPR-B

In order to characterize amino acids within the hPR
HBD which are critical for ligand binding and hormone-
dependent transactivation, libraries of mutated hPR
molecules were created and the mutants introduced into a
5   reconstituted progesterone-responsive transcription system
in yeast. This system allowed the screening of large
numbers of mutant clones and the direct, visual identifi-
cation of phenotypes.

Unique restriction sites for NaeI, AvrII and EcoNI
10   were created in the cDNA of hPR, obtaining three cassettes
of 396, 209 and 400 nucleotides (regions 1, 2 and 3,
respectively). For PCR mutagenesis three sets of primers
(16 + 7 for region 1, 5 + 4 for region 2 and 6 + 13 for
region 3) were used in the polymerization reaction using
15   YEphPR-B as DNA template. The fragments obtained after
PCR were digested with the appropriate enzymes, gel-
purified and ligated into the parental plasmid YEphPR-B.
Ligation mixes were used to transform bacterial cells and
to obtain libraries of hPR molecules randomly point-
20   mutated in the HBD. 5 μg of DNA were used from each
library to transform yeast cells carrying the reporter
plasmid YRpPC3GS+ and transformants were selected for
tryptophan and uracil auxotrophy on CAA plates containing
100 μM $CuSO_4$. These were then replicated on CAA plates
25   containing the hormones. The screening for "up-mutations"
allowed identification of receptor mutants with hormone-
independent transcriptional activity, or increased affini-
ty for the ligand (these clones should remain blue when
grown with 100-fold less hormone), or with an altered

response to RU486 or a glucocorticoid analogue. In the "down-mutation" screening, receptor mutants that were transcriptionally inactive in the presence of the ligand were detected.

5   Because of the nature of the method used to generate the mutated DNA templates, it was necessary, firstly, to determine the quality of the libraries obtained. This was assessed by estimating the number of null-mutations generated by mutagenesis. We estimated the frequency of

10 occurrence of transcriptionally inactive receptors (white colonies) compared to the total number of colonies. This frequency was about 7%.

The primary transformants were replica-plated onto plates containing the antiprogestin RU486. The wild type

15 receptor is not activated by this hormone (Fig. 1). Using this screening strategy, a single colony was identified that displayed considerable transcriptional activity in response to the antihormone. Interestingly, the same colony did not display transcriptional activity when

20 replica-plated in the presence of progesterone. The colony was purified and the phenotype was confirmed. Eviction of the expression vector from the clone, followed by reintroduction of the unmutated receptor, demonstrated that the phenotype was indeed related to the expression

25 vector and was not the result of a secondary mutation. In addition, the mutated plasmid called UP-1, was rescued from yeast by passage through *E.coli* (as described in Ward, *Nucl. Acids Res*. 18:5319 (1990)) and purified. This DNA was then reintroduced into yeast that contained only

the reporter plasmid.   As expected, the mutant phenotype
was stable and related directly to the receptor expression
plasmid.

                              EXAMPLE 11

 5   Characterization of the UP-1 Mutant

          The plate assays used to identify the receptor
mutants   are   qualitative   in   nature.   To   further
characterize the properties of UP-1, the activity of the
receptor mutants was compared with that of the wild type
10   receptor in a transcription assay.   In this method, yeast
cells transformed with either the wild type or the mutant
receptor and a progesterone responsive reporter were grown
overnight in the presence of 100μM $CuSO_4$.   When the cells
had reached an O.D.$_{600nm}$ of 1.0, they were supplemented with
15   progesterone or RU486 and harvested by centrifugation
after four hours. The β-galactosidase activity in the cell
cytosol was then measured.

          With reference to Figure 2, panel (A), when assayed
with the wild type receptor, 1 μM RU486 is a weak inducer
20   of transcription, whereas progesterone caused a greater
than 60-fold induction of transcription at 1 μM.   However,
this situation was reversed when the mutant was analyzed.
In this case, RU486 was an extremely potent activator,
whereas progesterone was ineffective.   Interestingly, the
25   activity achieved by the mutant in the presence of RU486
was of the same order of magnitude as that of the wild
type assayed in the presence of progesterone.   This
reversal in specificity clearly indicates that the

mechanism by which these ligands interact with the receptor is basically different.

Figure 2 shows the DNA and amino acid sequences of the wild type and mutant DNAs. The cytosine at position 2636 was missing in the mutant DNA, therefore, a shifted reading frame was created and a stop codon was generated 36 nucleotides downstream of the C-2636 deletion. A schematic structure of the wild type and UP-1 receptors is also presented with a depiction of the 12 C-terminal amino acids unique to the mutant receptor. Conserved and structurally similar amino acids are marked by an apostrophe and asterisk, respectively.

DNA sequence analysis of UP-1 identified a single nucleotide deletion at base 2636 (Fig. 2B). This mutation results in a shift of the reading frame which generates a stop codon 36 nucleotides downstream. As a result, the wild type receptor is truncated by 54 authentic amino acids and 12 novel amino acids are added at the C-terminus.

EXAMPLE 12

Western Analysis of the Mutant Human Progesterone Receptor

Figure 3 shows a western analysis of mutant hPR. Yeast cells carrying the reporter plasmid and wild type (yhPR-B or mutant (UP-1) hPR were grown overnight in CAA medium with (lanes 3 to 5 and 7 to 9) or without (lanes 2 and 6) 100 μM CuSO$_4$. 1 μM progesterone or 1 μM RU486 were added as indicated and cells were grown for another 4 hours. Yeast extracts were prepared as described above.

50 μg of protein extract were run on a 0.1% SDS-7% poly-
acrylamide gel.   50 μg of a T47D nuclear extract contain-
ing the A and B forms of hPR were also loaded (lane 1) as
a positive control.   The positions of molecular weight
5   markers are indicated.

A Western immunoblot analysis of UP-1 and wild type
receptors was performed in order to verify that the mutant
receptor was synthesized as predicted from its DNA se-
quence and to eliminate the possibility that some major
10   degradation products were responsible for the mutant
phenotype.   As shown in Fig. 3, the mutant receptor
migrated faster in the gel, confirming the molecular
weight predicted by DNA sequencing.   The wild type recep-
tor (yhPR-B) ran as a 114 kDa protein, while the mutant
15   receptor was 5kDa smaller (compare lanes 2 and 3 with 6
and 7).   The addition of 100μM CuSO$_4$ to the cell cultures
increased synthesis of both the wild type and mutant hPR
to the same extent.   No major degradation products were
detected.   In the presence of progesterone and RU486,
20   yhPR-B bands were upshifted due to hormone-induced phos-
phorylation of the receptor.   In contrast, RU486 induced
upshifting of wild type PR to a lesser extent (lanes 4 and
5).   For the UP-1 mutant this hormone-dependent upshifting
was seen upon treatment with RU486 (lanes 8 and 9).   Thus,
25   the C-terminus of PR may be responsible for the inactivity
of RU486.   Consequently, removal of this sequence would
enable RU486 to become an agonist.

EXAMPLE 13

SSSD/63950. v01

Hormone Binding Analysis

Figure 4 shows the transcriptional activity and hormone binding analysis of wild type and mutant hPR constructs. The hPR constructs are reported to the left side together with a schematic representation of the receptor molecules. Yeast cells were grown in the presence of 100 μM $CuSO_4$. Transcriptional analysis was done as described above. Experiments were done in triplicate and transcriptional activities were normalized with respect to protein. Hormone binding assays were performed in the presence of 20 nM [^{3}H] progesterone or 20 nM [^{3}H] RU486.

A saturation binding analysis of the UP-1 mutant receptor was performed in order to determine if its affinity for RU486 and progesterone was altered. Scatchard analysis of the binding data demonstrated that both the wild type and mutant receptors had a similar affinity for RU486 of 4 and 3 nM, respectively. As seen in Figure 4, the mutant receptor molecule had lost the ability to bind progesterone. Thus, the amino acid contacts for progesterone and RU486 with hPR are different.

EXAMPLE 14

Generation of Deletion Mutants of hPR-B

As shown in Fig. 2B, DNA sequencing revealed that the frameshift mutation in the UP-1 clone created a double mutation in the receptor protein. That is, a modified C-terminal amino acid sequence and a 42 amino acid trunca-

tion.   In order to identify which mutation was ultimately

responsible for the observed phenotype, two new receptor

mutants were constructed *in vitro*: YEphPR-B879, containing

a stop codon corresponding to amino acid 880, and YEphPR-

5   B891, containing a stop codon at amino acid 892.   Hormone

binding data (see Fig.4) demonstrated that both of these

truncated receptors could bind RU486 but not progesterone.

When examined *in vivo*, both mutant receptors activated

transcription   in   the   presence   of   RU486   to   levels

10   comparable to those of the mutant UP-1 generated in yeast.

As expected, both mutants were inactive in the presence of

progesterone.   Thus, the observed phenotype was not due to

second site mutations in the UP-1 molecule.   Also, 12

additional amino acids, from 880 to 891, were not respon-

15   sible for the mutant activity.   In addition, it is clear

the C-terminal 42 amino acids are required for progester-

one to bind to the receptor while the last 54 amino acids

are unnecessary for RU486 binding.   Thus, the antagonist

is contacting different amino acids in the native receptor

20   molecule and may induce a distinct receptor conformation

relative to agonists.

In addition to the above deletion mutations, other

deletions   in   the   C-terminal   amino   acid   sequence   have

provided   binding   activity   with   RU486   and   not   with

25   progesterone.   Such deletions include: (1) a 16 amino

acid   deletion   leaving   amino   acids   1-917   of   the

progesterone receptor; and (2) a 13 amino acid deletion

leaving amino acids 1-920 of the progesterone receptor.

Use   of   the   receptor   binding   region   with   TATA-CAT

expression in transient transfection assays showed CAT expression with the 16 amino acid deletion, *i.e.*, amino acids 640-917, and the 13 amino acid deletion, *i.e.*, amino acids 640-920.

5   EXAMPLE 15

Steroid Specificity for Activation of Transcription of the
UP-1 Mutant

Figure 5 shows the specificity of the transcriptional activity of the mutant hPR. In panel (A), wild type and
10 UP-1 mutant receptor transcriptional activities were assayed in the presence of different concentrations of progesterone, RU486, Org31806 and Org31376 as indicated.

A transcription assay was performed using two synthetic antagonists, Org31806 and Org31376, which are
15 potent antiprogestins. As shown in Fig. 5A, the mutant receptor was activated by both of these compounds. The curve of the concentration-dependent activity was similar to that obtained with RU486, suggesting that the affinity of these two antagonists for the mutant receptor is
20 similar to that of RU486. When assayed with the wild type receptor, these compounds had minimal transcriptional activity and behaved like partial agonists (3-10% of progesterone activity) only at concentrations of 1 μM, as does RU486. Thus, the inhibitory effect of the C-terminus
25 of hPR extends to other receptor antagonists.

In panel (B), transcriptional activities of wild type and UP-1 mutant receptors were assayed in the presence of 1 μM progesterone (P), RU486 (RU), R5020 (R), dexametha-

sone (D), cortisol (C), estradiol (E), tamoxifen (TX) or nafoxidine (N) (see Fig.5B). The synthetic agonist R5020 had no effect on the UP-1 mutant, suggesting that agonists, such as progesterone and R5020, require the C-

5   terminus of the native receptor for binding and consequently fail to recognize the truncated UP-1 receptor. Other steroids known to enter yeast cells, such as estradiol, the antiestrogens tamoxifen and nafoxidine, dexamethasone and cortisol, might possibly activate the

10   mutated receptor. All steroids tested were found to be inactive with either the wild type or mutant receptor. Thus, the activation of the mutant receptor is specific to antiprogestins.

EXAMPLE 16

15   Transcriptional Activity of Mutant Receptors in Mammalian Cells

Figure 6 shows the transient transfection of mutant hPR into mammalian cells. In panel (A), HeLa cells were transiently transfected with phPR-B and pHPR-B891 recep-

20   tors together with PRETKCAT receptor plasmid using the polybrene method of transfection as described (Tsai, et al. 1989). Cells were grown with or without 100 nM progesterone or RU486 for 48 hours prior to harvesting. CAT assays were performed as described above. In panel

25   (B), CV-1 cells were transiently transfected as in (A).

With reference to Figure 6, mutant receptor activity was assayed in both human endometrial HeLa cells and monkey kidney CV-1 fibroblasts. A mutant, phPR-891, was

constructed by replacing the full-length PR insert of phPR-B vector with the truncated PR cDNA of YEphPR-B891. The resulting receptor mutant, phPR-B891, is a 42 amino acid truncation of hPR-B form. Mutant 891 and wild type

5   receptors were transfected into HeLa cells together with the PRETKCAT reporter plasmid, which contains two copies of a GRE/PRE element.

As expected, wild type PR activated transcription of the CAT gene reporter in the presence of 10^{-7}M progesterone

10   (Fig. 6A). Although basal transcription level was high, a 3- to 4-fold induction of transcription was detected when progesterone was added to the media. In contrast, no induction occurred in the presence of RU486. The high basal level of transcription detected in these experiments

15   may mask or alter an RU486 effect on wild type hPR.

On the other hand, an induction of CAT activity was observed when the 891 mutant was incubated in the presence of 10^{-7}M RU486 (Fig. 6A). The same concentration of progesterone had no activity.

20   Cell-type specific factors can influence the activity of the transactivating domains of steroid receptors. In order to evaluate this possibility, wild type and mutant receptors were transfected into CV-1 cells. Similar results were obtained, *i.e.*, progesterone activated the

25   wild type receptor while RU486 activated 891 mutant receptor (Fig. 6B).

The protein synthesized from phPR-B891 plasmid was of the correct molecular weight in mammalian cells. The mutant receptor was transfected into COSM6 cells. Western

analysis on cell extracts showed that the 891 mutant was synthesized, as expected, as a protein of 109 kDa, which corresponds to a protein 42 amino acids shorter than the wild type hPR.   Thus, RU486 acts as an agonist of the

5   truncated B-receptor in a yeast reconstituted system and also in mammalian cells. The mechanism of transactivation does not require the C-terminal tail of the mutant receptor and is conserved between the three species tested.

EXAMPLE 17

10 <u>Construction of poly-glutamine stretch insertion into the LBD</u>

The poly-glutamine stretch containing multiple repeats of CAG was constructed by a method developed by S. Rusconi (Seipel et al., *Nucl. Acid Res.* 21:5609-5615)

15 utilizing multimerization of DNA fragment (BsaI and BbsI digested) coding glutamine repeats leading to poly-Q_n. Plasmid pBluscript-KS(II) was digested with Acc65I and SacI, the linearized vector was gel purified and ligated with the annealed oligonucleotide pair R3/R4 to create

20 plasmid pPAP. The oligonucleotide sequence for R3 (upper strand)   is: 5'-GTACGTTTAAACGCGGCGCGCCGTC GACCTGCAGAAGCTTACTAGTGGTACCCCATGGAGATCTGGATCCGAAT TCACGCGTTCTAGATTAATTAAGC-3' (Seq. ID No. 2)   and the sequence   for   R4   (lower   strand)   is: 5'-

25 GGCCGCTTAATTAATCTAGAACGCGTGAATTCGGATCCAGATCTCCATGGGG TACCACTAGTAAGCTTCTGCAGGTCGACGGCGCGCCGCGTTTAAAC-3' (Seq. ID No. 3).

        The following restriction sites are incorporated into

pPAP as the multiple cloning sites (from T3 to T7): PmeI,

AscI, SalI, PstI, HindIII, SpeI, Acc65I, NcoI, BglII,

BamHI, EcoRI, MluI, XbaI, PacI, NotI, SacI.

5   Oligonucleotides coding for 10 glutamines were annealed

and subcloned into the BglII and BamHI site of plasmid

pPAP.   The sequence for the upper and lower strand

oligonucleotide   are,   10QU   5'-

GATCTCGGTCTCCAACAGCAACAGCAACAGCAACAGCAACAGGGTCTTCTG-3'

10 (Seq.   ID   No.   4)   and   10QL: 5'-

GATCCAGAAGACCCTGTTGCTGTTGCTGTTGCTGTTGCT   GTTGGAGACCGA-3'

(Seq. ID No. 5), respectively.   The insert was confirmed

by restriction digestion and sequencing.

        The plasmid with 10Q insert (pPAP-10Q) was digested

15 with BsaI and BbsI (New England Biolab) overnight and

precipitated.   One   tenth   of   the   precipitated   DNA

(containing both vector and fragment) was religated to

create plasmid pPAP-18Q.   Each ligation step results in

pAP-2(n-1)Q from the previous vector pPAP-nQ.   In this way

20 various expansion of poly-Q was achieved and resulting

plasmids pPAP-34Q, pPAP-66Q and pPAP132Q were created and

confirmed by sequencing.   The BglII and BamHI fragment

(coding for poly-Q stretch) from these plasmids were

purified and cloned into BglII site of pRSV-GLVP to

25 generate GLVP with various poly-Q insert at the N-

terminus.   These GLVP-nQ were reinserted into the pCEP4

vector creating pCEP4-GLVP-nQ.

        Lengthening the C-terminal ligand binding domain from

879 to 914 (Figure 17), gradually increased RU486 induced

activation of target gene expression. Importantly, these
mutants responded specifically to RU486, but not to the
progesterone agonist R5020. Further extension of the C-
terminal LBD beyond aa 914 resulted in a decrease of GLVP
5   response to RU486.

Construction, Characterization and Analysis of mutant
Human GR-PR Fusion Protein Receptors

EXAMPLE 18

Plasmid Construction

10   A mutated human Progesterone Receptor was constructed
and characterized as discussed above. Mutagenesis of the
ligand binding domain of the human PR was carried out
under   the   same   procedures   outlined   above.
Characterization of the mutant progesterone receptor
15   identified a single nucleotide deletion at base 2636.
This mutation resulted in a shift of the reading frame
which generates a stop codon 36 nucleotides downstream.
As a result, the wild type receptor is truncated by 54
authentic amino acids and 12 novel amino acids are added
20   at the c-terminus. The 42 amino acid truncation to the c-
terminus was capable of binding RU486 and characterized as
discussed above.

        Plasmid DNA encoding the GR-PR fusion protein
receptor and the wild type GR were constructed as follows.
25   Each insertional mutant was digested with the restriction
enzymes BamH1 and Xhol, which flanked the 3' side of the
SV40 polyadenylation signal. The resulting fragments were

isolated from an agarose gel. The large fragment of the insertional mutant containing the amino-terminal coding portion of the GR, *i.e.*, the transregulatory and DNA binding region, and the bulk of the plasmid were ligated

5   with the small fragment of another insertional mutant containing the carboxyl terminal coding sequence of the hPR deletion mutant prepared above. The resulting plasmids carrying the deletion in the hPR ligand binding domain were sequenced to ensure the integrity of the GR-PR

10 mutant constructs.

In addition, plasmid DNA encoding a mutated rat or human GR and the wild type rat or human GR were also constructed. The plasmids for rat pGR0385 (or prCS1.C) and its wild type pGR0384 were constructed using the above

15 methods. Details regarding construction, mutation and characterization of the above plasmid can be found in Lanz and Rusconi, *Endocrinology* 135:2183-2195 (1994), all of which is hereby incorporated by reference, including drawings. Characterization of the rat and human mutant GR

20 identified a double point mutation in the ligand binding domain. In the rat construct, amino acids 770, 771, methonine and leucine, were substituted with alanine and alanine. Amino acids 780 and 781 were deleted. In the human constructs, amino acids 762 and 763 were deleted.

25 Amino acids 752 and 753 were substituted with alanines. Both the substitution and deletion changes were at the carboxyl terminus portion of the rat or human GR ligand binding domain. The insertional mutant was digested with the restriction enzymes BamH1 and Xhol, which flank the

3'side of the SV40 polyadenylation signal and the
resulting fragment was isolated from agarose gel. The
large fragment of one insertional mutant containing the
amino-terminal coding portion of the rat or human GR and
5   the bulk of the plasmid were ligated with the small
fragment of another insertional mutant containing the
carboxy-terminal coding sequences of the mutated ligand
binding domain. The resulting plasmids carrying the
deletion in the ligand binding domain were sequenced to
10 ensure the integrity of the rat or human GR mutants.

In addition, the above procedures were also used to
construct plasmid DNA encoding a GR mutant with a
constitutively active receptor, *i.e.*, pGR0403R (Figures 9
and 10). The insertional mutant was digested with the
15 appropriate restriction enzyme. The resulting fragments
were isolated from agarose gel. The large fragment of the
insertional mutant containing the amino-terminal coding
portion of the GR, *i.e.*, the transregulatory domains and
DNA binding domains, and the bulk of the plasmid were
20 ligated with the small fragment of another insertional
mutant containing the mutated GR ligand binding domain.
The resulting plasmid was sequenced (Figure 9) to ensure
integrity of the mutant construct.

EXAMPLE 19

25 Cell Culture, Transfection and Assay of CAT and Luciferase
Activities

CV-1 cells were maintained at 37°C in Dulbecco
modified Eagle medium containing 10% fetal bovine serum

("FBS") in a humidified atmosphere containing 5% CO_2. Cells were transfected using the commercially available cationic agent lipofectamine. Briefly, DNA was mixed with the lipofectamine reagent and added to cells. After 5 hours, the DNA mix was removed and replaced with growth medium containing 10% FBS and cells were returned to an atmosphere containing 5% CO2. Eighteen hours later, cells were treated with steroids at various concentrations for approximately 24 hours, then harvested.

In this method, the CV1 cells are transformed with either the wild-type receptor or the mutant receptor and a glucocorticoid responsive reporter construct. To measure transcriptional activation, a CAT reporter containing two synthetic GRE's and a TATA box was used. To measure transcriptional repression, two constructs were used. The first contains two copies of the binding site for the inflammation-inducible transcription factor AP-1, following by the thymidine kinase (tk) promoter, linked to CAT. The second contains two copies of the binding site for the inflammation-inducible transcription factor NF_K-B, followed by a TATA box, linked to the luciferase gene. CAT expression was quantified using an ELISA assay following the manufacturer's recommended procedure. Luciferase activity was measured using a commercially-available luciferase assay following the manufacturer's recommended procedure.

EXAMPLE 20

In vitro Transfections Using CV1 Cells

The GR-PR fusion protein receptor and the mutant rat GR were tested for biological activity through in vitro transfection into CV1 cells.   As controls vectors expressing the wild type human GR and the wild type rat GR

5   were used.   Results from these experiments demonstrate that the wild type human and wild type rat GR are transcriptionally activated in response to dexamethasone and minimally by RU486.   In contrast, the mutant rat GR (CS1.CD) is transcriptionally activated by RU486 and not

10   by dexamethasone.   Similarly, the GR-PR fusion protein receptor is also activated by RU486 and not by dexamethasone.   Figure 11 illustrates the amount of CAT protein produced in response to the particular ligand.

<u>EXAMPLE 21</u>

15   <u>In vitro Transcriptional Repression Studies</u>

The transcriptional repression mediated by the mutant rat GR and human GR-PR construct were examined.   The amount of CAT protein produced under the transcriptional control of synthetic activation elements was determined.

20   Specifically two reporters were examined TRE2tkCAT, which contains AP-1 fused to the thymidine kinase promoter linked to CAT. The second reporter used was NF_K-B-luc plasmid, which contains 2 NF_K-B binding sites fused to luciferase.   These promoters contain inflammation-

25   inducible promoters, and were used to evaluate the ability of the wild-type and mutant GR constructs to repress transcription.

Cells were transfected into CV1 cells along with either the wild type rat or human GR or the mutant rat (CS1.CD) or human GR. Cells pretreated with dex or RU486 to allow binding to the steroid receptor, were then

5   stimulated with phorbol ester TPA to activate AP-1 and NF_K-B. Companion cells were not stimulated with TPA, and control cells also received neither dex nor RU486.

The results demonstrate that RU486 treatment resulted in a decrease in the level of CAT protein and luciferase

10   activity in CSI.CD transfected cells. Dex treatment had no effect on CAT levels or luciferase. These results were not expected since dex does not bind to the ligand binding domain of the mutant rat GR CSI.CD or human GR. In cells transfected with the wild type GR both dex and RU486

15   caused a decrease in the level of CAT protein and luciferase activity. Such results are not unexpected because the wild type GR binds both dex and RU486.

RU486 acts through the mutated GR to repress transcription of AP1 driven genes. Since AP-1 and NF_K-B

20   drive expression of pro-inflammatory genes, and RU486 acts through mutant or represses transcription of the AP-1 and NF_K-B driven genes, there was mediation of the anti-inflammation.

EXAMPLE 22

25   Mutant GR Expression and Detection

Three antibodies were obtained and used to recognize recombinant partially purified GR in a Western blot analysis. Studies were performed to detect wild type GR

and mutant GR protein from transfected cells or GR from rat synovial tissue using the above antibodies.

The antibodies also were able to detect human GR obtained from HeLa cell extracts. Significant levels of
5   GR were detected with as low as 200ug of whole cell extract. Immunoreactivity was also detected with synovial tissue, and antibodies are being prepared to distinguish between wild type and mutant GR proteins.

EXAMPLE 23

10 <u>Transactivation and Transrepression Studies</u>

In addition to the experiments above, the vector with NF_K-B binding sites fused to the luciferase gene, was injected into synovial joints in rats and treated with and without TNF-α. TNF-α is a cytokine which induces
15 inflammation and promotes NF_K-B binding to its appropriate DNA sequences. With the DNA construct, TNF-α treatment results in an increase in transcription of TNF-α and exogenously-introduced luciferase gene. No luciferase activity in synovial tissue is detected without plasmid
20 transfection. Also, there is no luciferase activity in synovial tissue injected with plasmid in the absence of TNF. A six-fold increase in the level of luciferase occurred when tissue was exposed to 0.1 or 1nM TNF. This serves as an easily detectable in vivo marker for wild-
25 type or mutant GR function.

Construction, Characterization and Analysis of
Double Point Mutations in the Ligand Binding Domain of GR

EXAMPLE 24

Mutagenesis of the ligand binding domain of human GR

5   A plasmid was constructed containing the human GR
cDNA with amino acids 752 and 753 changed to alanines and
amino acids 762 and 763 deleted. This plasmid, pSTC-hGR-
CS1/CD, was constructed as follows. The wild type
glucocorticoid hormone receptor plasmid was digested with
10 the restriction enzymes NsiI and XbaI, which flank the
region to be mutated. The resulting fragments were
isolated from agarose gel. The smaller fragment was
digested with the restriction enzymes EcoRI and SspI,
generating three fragments. The fragments were isolated
15 from an agarose gel.

        A synthetic fragment was synthesized: 5'-AAT TCC CCG
AGG CGG CAG CTG AAA TCA TCA CCA ATC AGA TCT-3' (Seq. ID
No. 6) to replace the EcoRI-SspI fragment. The larger
plasmid fragment, the NsiI-EcoRI fragment, the SspI-XbaI
20 fragment and the synthetic EcoRI-SspI fragment were
ligated together. The resulting plasmid carries the
substitution and deletion as described above.

EXAMPLE 25

Characterization of GR Mutants in the Ligand Binding
25 Domain

        To ensure the integrity of the mutation, the plasmid
containing the mutant human GR was sequenced. Further

experiments, as discussed above, were done to characterize the mutant human GR. Western analysis and hormone binding as discussed above were performed to ensure character of the constructs, *e.g.*, cell expression of the protein and

5 steroid specificity for activation or repression of transcription.

EXAMPLE 26

Transcriptional Activity of the Mutant Receptors in Mammalian Cells

10   LMTK⁻ cells were maintained at 37°C in Bulbecco's modified Eagle's medium containing 10% fetal Bovine serum ("FBS") in a humidified atmosphere containing 5% CO_2. Cells were transfected with the polybrene method described in Kawai et al., *Mol. Cell. Bio.* 4:91-1172 (1984), hereby

15 incorporated by reference, including drawings. After a 25% glycerol shock in Hank's buffered saline solution ("HBSS"), the cells were washed twice with HBSS and medium was added containing hormones or solvent. The cells were cultured for 48 hours. Extracts were made by freeze-

20 thawing. CAT activity was assayed with 25 μg protein and an incubation time of 16 hours. CAT activity assayed as described by Seed et al., *Gene* 67:271 (1988), hereby incorporated by reference, including drawings.

Construction, Characterization and Analysis of

25   ### Constitutively Active Mutant GR

EXAMPLE 27

Mutagenesis of the ligand binding domain of human GR

Deletion of the steroid ligand binding domain was prepared as follows. This deletion removed a large portion of the carboxyl-terminal portion of the protein eliminating all steroid binding properties. Using the

5   procedures discussed above, the pGR0403R plasmid (Figures 9 and 10) was constructed. This mutation gave rise to a constitutively active receptor. This mutant was able to activate transcription of the CAT reporter gene in the presence or absence of glucocorticoid hormone. In

10 addition, this mutant is also able to repress transcription of the NF_K-B-luciferase construct.

EXAMPLE 28

Characterization of GR Mutants in the Ligand Binding Domain

15 To ensure the integrity of the mutation, the plasmid containing the mutant human GR, pGR0403R (Figure 10) was sequenced (Figure 9). Further experiments, as discussed above, were done to characterize the mutant human GR. Western analysis and hormone binding as discussed above

20 were performed to ensure character of the constructs, e.g., cell expression of the protein, lack of steroid specificity for activation or repression of transcription and base level of gene expression as compared to constitutive expression.

EXAMPLE 29

Transcriptional Activity of the Mutant Receptors in Mammalian Cells

        The constitutively active mutant GR construct was
5 prepared as discussed above. The receptor has no ligand
binding domain and, when expressed in cells, represses
transcription of AP-1 driven genes in the absence of dex
or RU486. In vitro testing shows that the constitutively
active GR mutant when transfected constitutively activates
10 promoters with glucocorticoid responsive elements and
represses AP-1 containing promoters.

Construction, Characterization and Analysis
of Mutations in the DNA Binding or Transregulatory
Domains of GR

15   EXAMPLE 30

Mutagenesis of the DNA Binding or Transregulatory Domains of GR

        For obtaining transactivation activity without
transrepression activity the following construct was
20 made. The mutated ligand binding domain is mutated as
described above. Procedure details from Lanz, et al.,
Endocrinology 135:2183-2195 (1994) are hereby incorporated
by reference, including drawings. The mutated DNA binding
domain is mutated by changing the serine at position 425
25 to glycine, the leucine at position 436 to valine and the

tyrosine and asparagine at positions 478 and 479 to leucine and glycine.

For obtaining transrepression activity without transactivation, the following construct was made. The 5 mutated ligand binding domain is mutated as described above. The mutated transregulatory domain is mutated by changing the alanine at position 458 to threonine, the asparagine and alanine at positions 454 and 458 to aspartic acid and threonine, respectively, and the 10 arginine and aspartic acid at positions 460 and 562 to aspartic and cysteine, respectively.

EXAMPLE 31

Characterization of GR Mutants in the DNA Binding or Transregulatory Domains

15   To ensure the integrity of the mutation, the plasmids containing the mutant GR were sequenced. Further experiments, as discussed above, were done to characterize the mutant GR constructs. Western analysis and hormone binding as discussed above were performed to ensure 20 character of the constructs, *e.g.*, protein expression in cells and steroid specificity for activation or repression of transcription.

EXAMPLE 32

Transcriptional Activity of the Mutant Receptors in 25 Mammalian Cells

The above mutant GR constructs were prepared. The two different receptor constructs have either a mutated

DNA binding domain or a mutated transregulatory domain. When expressed in cells, the transrepression only construct with a DNA binding domain mutation represses transcription of AP-1 and NF_κ-B driven genes in the

5 presence of dex or RU486. No activation of transcription was observed. *In vitro* testing shows that the GR mutant when transfected represses AP-1 and NF_κ-B containing promoters and does not activate the glucocorticoid responsive genes.

10 As for the transactivation only construct with a mutated transregulatory domain, activation of transcription was observed in the presence of various steroids. In the presence of dex or RU486 no transrepression of AP-1 or NF_κ-B driven genes was detected.

15 *In vitro* testing shows that the GR mutant when transfected activates glucocorticoid responsive genes in response to ligand stimulation but no repression of AP-1 or NF_κ-B genes was observed.

EXAMPLE 33

20 Chicken, Rat and Mammalian Progesterone Receptors

Chicken, rat and mammalian progesterone receptors are readily available and function by binding to the same DNA regulatory sequence. Chicken and rat progesterone receptors, however, bind a different spectrum of ligands,

25 possessing affinities different from those interacting with human progesterone receptor. Thus, the chicken and rat progesterone receptor can be used as a transgene regulator in humans. Further, it can be used to screen

for specific ligands which activate chicken or rat progesterone receptor but not endogenous human progesterone receptor. An example of a ligand is 5α-pregnane-3,20-dione (dihydroprogesterone) which binds

5   extremely well to chicken and rat progesterone receptor but does not bind or binds very poorly to human progesterone receptor.

Although the unmodified chicken or rat progesterone receptors are already endowed with a different spectrum of

10 ligand binding affinities from the human or other mammals and can be used in its native form, it is important to try to select additional mutated progesterone receptor to create a more efficacious receptor. The differences in chicken, rat and human progesterone receptors are due to

15 a few amino acid differences. Thus, other mutations could be artificially introduced. These mutations would enhance the receptor differences. Screening receptor mutations for ligand efficacy produces a variety of receptors in which alterations of affinity occur. The initial

20 screening of progesterone mutants was carried out using intermediate levels of ligands. One mutant had lost progesterone affinity entirely, but bound a synthetic ligand RU486 with nearly wild-type efficiency. RU486 is normally considered an antagonist of progesterone

25 function, but had become an agonist when tested using this specific mutant. Because the ligand is synthetic, it does not represent a compound likely to be found in humans or animals to be treated with gene therapy. Although RU486 works as an agonist in this case, it is not ideal because

of its potential side effects as an anti-glucocorticoid. Further, it also binds to the wild-type human progesterone. Thus, it has the undesirable side effect of reproductive and endocrine disfunction.

5   This approach is not limited to the progesterone receptor, since it is believed that all ligand activated transcription factors act through similar mechanisms. One skilled in the art recognizes that similar screening of other members of the steroid superfamily will provide a

10   variety of molecular switches. For example, the compound 1,25-dihydroxy-Vitamin D_3 activates the Vitamin D receptor but the compound 24,25-dihydroxy-Vitamin D does not. Mutants of the Vitamin D receptor can be produced which are transcriptionally activated when bound to 24,25-

15   dihydroxy-Vitamin D, but not by 1,25-Vitamin D_3.

One skilled in the art recognizes that the ligands are designed to be physiologically tolerated, easily cleared, non-toxic and have specific effects upon the transgene system rather than the entire organism.

20   EXAMPLE 34

Transgenic Animals

        A modified glucocorticoid receptor can be used in the production of transgenic animals. A variety of procedures are known for making transgenic animals, including that

25   described in Leder and Stewart, U.S. Patent No. 4,736,866 issued April 12, 1988, and Palmiter and Bannister, *Annual Review of Genetics*, 20:465-499. For example, the mutated glucocorticoid receptors described above can be combined

with the nucleic acid cassette containing the recombinant gene to be expressed. For example, lactoferrin can be placed under the control of a basal promoter, such as thymidine kinase promoter with adjacent glucocorticoid

5 responsive elements. This vector is introduced into the animal germ lines, along with the vector constitutively expressing the mutant glucocorticoid receptor. The two vectors can also be combined into one vector. The expression of the recombinant gene in the transgenic animal is

10 turned on or off by administering a pharmacological dose of RU 38486 to the transgenic animal. This hormone serves to specifically activate transcription of the transgene. The dose can be adjusted to regulate the level of expression. One skilled in the art will readily recognize that

15 this protocol can be used for a variety of genes and, thus, it is useful in the regulation of temporal expression of any given gene product in transgenic animals.

Location of Transregulatory Domains at the C-Terminal

EXAMPLE 35

20 Chimeric Fusion Protein with Various C-terimnus Deletion

To construct GLVP chimeras with various C-terminal deletions of the human progesterone receptor ligand binding domain, the HindIII to BamHI fragment containing these various deletions in pRSV-hPR plasmids (Xu et al.

25 (1996) (unpublished)) was gel purified with QIAEX II gel extraction kit (Qiagen). The purified fragments were subcloned into HindIII and BamHI sites of pRSV-GLVP (Wang

et al., *Proc. Natl. Acad. Sci.* 91:8180-8184 (1994))
replacing the amino acid region 610 to 891 of the GLVP.

EXAMPLE 36

GLVP$_c$. Chimeras with VP16 Activation at the C-terimnus

5   Two-step clonings were used to move VP16 activation
to the C-terminus of the chimeric fusion protein. First,
the hPR -LBD region (from amino acid 800 to various C-
terminus) was amplified using 5' primer (5'-
TATGCCTTACCATGTGGC-3' (Seq. ID No. 7)) with a different 3'
10 primer as a pair and digested with HindIII to SalI to
prepare the fragment for ligation.   For a different
position of amino acid truncation, the 3' primers
incorporating the SalI site are: P3S-879: 5'-
TTGGTCGACAAGATCATGCATTATC-3' (Seq. ID No. 9); P3S-891: 5'-
15 TTGTCGACCCGCAGTACAGATGAAGTTG-3' (Seq. ID No. 10) and P3S-
914: 5'-TTGGTCGACCCAGCAATAACTTCAGACATC-3'.   The DNA
fragment containing the VP16 activation domain (amino acid
411-490) was isolated from pMSV-VP16-Δ3'-β58N' with SalI
and BamHI.

20   The digested PCR fragment and VP16 activation were
ligated together into the HindIII and BamHI sites of
expression vector pCEP4 (Invitrogen). The ligated vector
pCEP4-PV (LBD 810-879 and VP16), -C3 (LBD 810-891 and
VP16), -C2 (LBD 810-914 and VP16), respectively, now
25 contain C-terminal fragments of hPR-LBD from the HindIII
site (amino 810) to various truncations of LBD fused 3' to
VP16 activation domain with BamHI after the termination
codon of VP16. The HindIII-BamHI fragment from pGL (in

pAB vector) was then replaced with PV, C3, and C2
fragment, respectively, to yield pGL$_{879}$VP$_{C'}$, pGL$_{891}$VP$_{C'}$, and
pGL$_{914}$VP$_{C'}$. These chimeric fusion proteins were then
subcloned into Acc65I and BamHI sites of pCEP4 expression
5 and were named as pCEP4-GL$_{879}$VP$_{C'}$, pCEP4-GL$_{89}$VP$_{C'}$, pCEP4-
GL$_{914}$VP$_{C'}$ (Fig. 17).

The regulator with a C-terminally located VP16 is
more potent than its N-terminal counterpart (Fig. 18). In
addition, extension of the C-terminal LBD from amino acid
10 879 to amino acid 914 further increased transactivational
activity of the regulator in this C-terminally located
VP16 chimera. Thus, extension of the LBD to amino acid
914 further enhances the RU486-dependent transactivation,
irrespective of whether VP16 is located in the N- or C-
15 terminus, suggesting the existence of a weak dimerization
and activation function between amino acid 879 and 914 of
the PR-LBD. By transferring the VP16 activation domain
from the N-terminus to the C-terminus, a much more potent
transactivator GL$_{914}$VP$_{C'}$ was generated.

20   The modified GL$_{914}$VP$_{C'}$ is not only more potent but
also activates the reporter gene at a lower concentration
of ligand as compared to GL$_{914}$VP where VP16 is located at
the N-terminus. GL$_{914}$VP activity occurred at an RU486
concentration of 0.1 nM and reached a maximal level at 1
25 nM.   In contrast, GL$_{914}$VP$_{C'}$ increased reporter gene
expression at an RU486 concentration 10 fold lower (0.01
nM) than that of GL$_{914}$VP. This newly discovered character
of GL$_{914}$VP$_{C'}$ is important for its use in inducible target
gene expression, since it would allow use of a

concentration which has no anti-progesterone or anti-glucocorticoid activity. This represents a significant advantage when the inducible system is applied in *in vivo* situations, as exemplified by transgenic mice and gene
5   therapy.

                          EXAMPLE 37

Inducible Repressor Containing the Kid-1 KRAB domain

        The Kid-1 gene containing the KRAB domain (aa. 1-70) was amplified with 2 sets of primers for insertion into
10   the N- or C-terminus of GL_{914}, respectively. For the KRAB domain to be inserted at the N-terminus of the fusion protein, the Kid-1 cDNA was amplified with the set of primers as follows: Kid3: 5'-CGACAGATCTGGCTCCTGAGCAAAGAGAA-3' (Seq. ID No. 11), Kid4:
15   5'-CCAGGGATCCTCTCCTTGCTGCAA-3' (Seq. ID No. 12). The PCR products were digested with BglII and BamHI and subcloned into pRSV-GL_{891} to create pRSV-KRABGL $_{891}$ The KpnI-SalI fragment of KRABGL$_{891}$ was then purified and subcloned into KpnI-SalI sites in pRSV-GL_{914}VP to create pRSV-KRABGL$_{914}$.
20   The entire KRABGL$_{914}$ fragment (KpnI-BamHI) was then inserted into the KpnI and BamHI digested pCEP4 generating pCEP4-KRABGL$_{914}$ (Fig. 19).

        For C-terminally located KRAB domain, the Kid-1 gene was amplified with the following set of primers: Kid1:
25   5'-TCTAGTCGACGATGGCTCCTGAGCAAAGAGAAG-3' (Seq. ID No. 13), Kid2: 5'-CCAGGGATCCTATCCTTGCTGCAACAG (Seq. ID No. 14). The primer Kid2 also contains a termination codon (TAG) after aa. 70. The PCR products were digested with SalI and

BamHI and purified using QIAEX II gel extraction kit (Qiagen). The HindIII and SalI fragment (317 bp) from pBS-GL$_{914}$VP$_{C'}$, was isolated as is the vector fragment of pCEP4-GL$_{914}$VP$_{C'}$ digested with HindIII and BamHI. These

5   three piece fragments were ligated to create pCEP4-GL$_{914}$KRAB.

The chimeric regulator GL$_{914}$KRAB, with the KRAB repression domain inserted in the C-terminus, strongly repressed expression (6-8 fold) of both reporters in an

10   RU486-dependent manner. However, the N-terminally located KRAB repression domain (KRABGL$_{914}$) did not repress target gene expression in the presence of RU486 to the degree of that achieved with KRAB located in the C-terminus (GL$_{914}$KRAB).

15   EXAMPLE 38

Transient Transfection, CAT Assay , hGH assay and Western Blot

HeLa and CV1 cells were transfected with the described amount of DNA using the polybrene mediated Ca$_2$PO$_4$

20   precipitation method and CAT assay was performed and quantified as described above (Wang et al., *Proc. Natl. Acad. Sci.* 91:8180-8184 (1994)). HepG2 cells (10^6) were grown in DMEM with 10% fetal bovine serum and 1X Penicillin-Streptomycin-Glutamine (Gibco BRL) and

25   transfected with polybrene mediated Ca$_2$PO$_4$ precipitation method. Aliquots of the cell culture media were taken at different time intervals and hGH production was measured using the hGH clinical assay kit (Nichols Institute)

according to the manufacture's instruction. For Western blot analysis, protein extracts (20 μg) were prepared from transiently transfected HeLa cells, separated on SDS polyacrylamide gel and trans-blotted onto nylon membrane as described above. The blot was probed with anti-GAL4-DBD (aa. 1-147) monoclonal antibody (Clonetech) and developed with an ECL kit (Amersham).

These analyses confirmed that the two regulator proteins are expressed at a similar level. Together, these results suggest that through modification of the PR-LBD within the chimeric regulator we could further improve its response to a ligand by at least one order of magnitude.

EXAMPLE 39

Stable Cell Line Generation and Neurite Outgrowth Assay

To demonstrate the use of the inducible system in a biological situation, a regulatable expression model for nerve growth factor (NGF) was designed. NGF has been shown to stimulate neurite (axon) outgrowth of PC12 cells (from rat adrenal pheochromocytoma) when added to the cell culture media (Greene et al., *Proc. Natl. Acad. Sci.* 73:2424-2428 (1976)).

Rat FR cells, derived from rat fetal skin cells (American Type Culture Collection, CRL 1213) were transfected with pCEP4-GLVP$_{914}$VP$_{c'}$ by the Ca$_2$PO$_4$ method as described previously (Wang et al., *Proc. Natl. Acad. Sci.* 91:8180-8184 (1994)). Cells were grown in DMEM with 10% fetal bovine serum and selected with 50 μg/ml hygromycin-B

(Boehringer Mannheim). After 2-3 weeks colonies were picked and subsequently expanded. Each clone was then transiently transfected with 2 μg of the p17X4-TATA-CAT plasmid utilizing Lipofectin (GIBCO-BRL). Twenty-four

5   hours later, the cells were treated with either RU486 (10^{-8}M) or 80% ethanol vehicle. Cells were harvested 48 hours later and CAT activity was measured using 50 μg of cell extracts. Clones showing RU486 inducible CAT activity were subsequently transfected with the vector p17X4-TATA-

10   rNGF(Neo).

Stable cells containing both genes were selected with hygromycin (50 μg/ml) and G418 (100 μg/ml) for 2-3 weeks and subsequently expanded. Each colony was then seeded into a 10 cm culture dish and treated with 10^{-8}M RU486 or

15   vehicle control (80% ethanol). After 48 hours, the conditioned media was collected and frozen. Subsequently, the conditioned media was thawed and diluted two-fold in DMEM with 10% horse serum and 5% fetal bovine serum. The diluted conditioned media was then placed on PC12 cells,

20   with new diluted conditioned media added every two days After 5-7 days, PC12 cells were observed for neurite outgrowth.

When conditioned media (from C4FRNGF cells treated with RU486) was added to PC12 cells, strong neurite

25   outgrowth from PC12 cells was observed after 48 hrs of incubation. Little if any neurite outgrowth was observed in PC12 cells incubated with the conditioned media that was collected from stable cells treated with vehicle only (85% ethanol). These results demonstrate that the

inducible system can be used to control various biological phenomenon.

Mutated Glucocorticoid Receptors as Gene Switch

In addition to the above methods, the mutated
5 glucocorticoid receptors can be used as gene switches as described in U.S. Serial No. 07/939,246, by Vegeto et al., filed September 2, 1992, entitled "Mutated Steroid Hormone Receptors, Methods for Their Use and Molecular Switch for Gene Therapy," the whole of which (including drawings) is
10 hereby incorporated by reference. The above constructs of the present invention can be used to express a co-transfected target therapeutic gene using a glucocorticoid response element ("GRE") containing promoter. The GRE promoter will drive, activate or transactivate expression
15 of the therapeutic gene upon activation of the ligand binding domain of the constructs of the present invention. The therapeutic protein can be a secreted protein, *e.g.*, an anti-inflammatory cytokine. Such methods allow more global effect on the transfected tissue.

20 One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The mutated steroid receptors along with the methods, procedures, treatments,
25 molecules, specific compounds described herein are presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will

occur to those skilled in the art which are encompassed within the spirit of the invention are defined by the scope of the claims.

It will be readily apparent to one skilled in the art
5   that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

All patents and publications mentioned in the speci-fication are indicative of the levels of those skilled in
10 the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specif-ically and individually indicated to be incorporated by reference.

15   Other embodiments are within the following claims: